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(54) Title: NOVEL CHELATORS AND COMPOSITIONS PREPARED THEREWITH

(57) Abstract

Novel chelators and methods of synthesis and use thereof are disclosed. The compositions, methods and kits provided enable radionuclides for medical applications to be complexed to chelator-conjugates under mild conditions.

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NOVEL CHELATORS AND COMPOSITIONS PREPARED THEREWITH

Background of the Invention

Methods for labeling medically important macromolecules with metal radionuclides have been developed, but they have not yet proved generally useful because of limitations in various aspects of their use. In general, the practitioner chooses a particular macromolecule, e.g., a protein or nucleic acid, on the basis of its affinity for a particular target in a biological system, and this affinity is the basis for that molecule's potential as a therapeutic or diagnostic composition. The labeling method should preferably preserve the native structure of that molecule to assure that the binding function is not substantially reduced. Further, the potential use is enhanced if the radionuclide as supplied to the macromolecule is not substantially further transferred to miscellaneous cells or serum proteins, causing diminishment of radioactivity delivered to a target site, and increased non-specific background radiation. The ability to retain the radiolabel is a function of several factors, including the stability of the macromolecule to endogenous enzymes, possible chemical compromise to its structural integrity following the labeling procedure, and the nature of the chemical bond between the macromolecule and the radiolabel.

The N-[N-[N-[(benzoylthio)acetyl]glycyl]glycyl]glycine (MAG_3) chelator of ^{99m}Tc was originally developed as an alternative to radiolabeled hippuran for renal function studies (Fritzberg AR., Kasina S., Eshima D., Johnson D.L., *J. Nucl. Med.*, 27: 111-116; 1986). This succinimide ester mercapto-acetyl tripeptide is protected against disulfide-bond formation by a benzoyl group, which must be heated to 100°C for 10 min during labeling to remove the protecting group. This benzoyl-protected chelator has also been used to radiolabel antibodies with ^{99m}Tc (Fritzberg A.R., Berninger R.W., Hadley S.W. *et al.*, *Pharmaceutical Res.*, 5: 325-334; 1988) and radiorhenium (Goldrosen MH., Biddle WC., Pancook S. Bakshi S., Vanderheyden J-L., Fritzberg A.R., Morgan A.C., Foon K.A., *Cancer Res.*, 50: 7973-7978; 1990). However, past use of the chelator for protein labeling has been limited since the benzoyl protecting group requires extreme alkaline pH or boiling temperatures for sulfur deprotection. The MAG_3 chelator has also been used to label antibodies by post-conjugation methods through the use of an isophthaloyl group for protection in place of the benzoyl group (Weber R.W., Boutin R.H., Nedelman M.A., Lister-James J., Dean R.D., *Bioconjug. Chem.* 1:431-437, 1990). However, in addition to a complicated synthesis, this approach requires an additional step (deprotection) and the immediate labeling of the deprotected-conjugated antibody before disulfide bond formation can occur in solution. Accordingly, this chelator has

been radiolabeled prior to conjugation (i.e., preconjugation labeling) with macromolecular polymers such as proteins or polypeptides, which cannot withstand harsh conditions. Preconjugation labeling can be a complex procedure with multiple intermediate purification steps. More importantly from the point of view of application of radionuclide-labeled macromolecules for diagnostics, imaging and therapeutics, preconjugation labeling can limit the usefulness of the product: the pre-conjugation radionuclide-chelator complex frequently has a short half-life, cannot be transported without necessary precautions for radioactivity, and can expose end-users to radioactivity during a number of complex synthetic steps, all required prior to end use with samples or patients. Ram and Buchsbaum (Cancer Suppl. 73:769-773, 1994) disclose the labeling of antibodies with chelators, however these require a specially modified phenylalanine residue n the chelator to accomplish conjugation.

Oligonucleotides and polynucleotides are of interest for development of new products for diagnostics, imaging and therapy, due to their ability to hybridize specifically to oligonucleotides of complementary sequence. This property of single-stranded oligomers (i.e., to locate the complementary sequence and form a double-stranded molecule or a complex as a third strand) can be used to advantage in radiopharmaceutical development. For example, oligonucleotide DNAs are currently under investigation for antisense applications (Uhlmann E., Peyman A., *Chem. Rev.*, 90: 543-584; 1990; and Dewanjee M.K., *Diagn. Oncol.*, 3: 189-208, 1993.). If radiolabeled, these oligonucleotides may usefully deliver radioactivity to targeted cells or tissues. Recently, c-myc oncogene mRNA was targeted in mice with a radiolabeled antisense probe (Dewanjee M.K., Ghafouripour A.K., Kapakvanjwala M., Dewanjee S, Serafini AN, Lopez DM, Sfakinakis GN. *J. Nucl. Med.*, 35: 1054-1063, 1994). Other possible applications include methods of radiolabeling large molecules by hybridization, pretargeting approaches based on oligonucleotides (Kuijpers WHA, Bos ES, Kaspersen FM, Veeneman GH, van Boeckel CAA., *Bioconj. Chem.*, 4: 94-102; 1993) and the amplification of radioactivity within a tumor or other lesion by sequential administration of complementary DNAs (Hnatowich D.J., Winnard P. Jr., Virzi F. Fogarasi M., Sano T, Smith CL, Cantor CR, Rusckowski M., *Proceedings, Fifth Conference on Radioimmunodetection and Radioimmunotherapy of Cancer*, Princeton NJ., 1994).

Proteins have been labeled with technetium-99m (^{99m}Tc) using the hydrazino nicotinamide (SHNH) chelator (Abrams M.J., Juweid M., tenKate C.I., Schwartz D.A., Hauser M.M., Gaul F.E., Fuccello A.J., Rubin R.H., Strauss H.W., Fischman A.J, *J. Nucl. Med.*, 31: 2022-2028, 1990) and the label found to be stable *in vitro* and *in vivo* (Hnatowich D.J., Mardirossian G., Ruscowski M., Fogarasi M, Virzi F, Winnard P Jr., *J.*

Nucl. Med., 34; 109-119, 1993). The SHNH chelator was initially used for oligonucleotides, however, transfer of label nonspecifically to proteins from oligonucleotides labeled in this manner was observed. The identical oligonucleotide, radiolabeled with ^{111}In using the chelator diethylenetriamine-pentaacetic acid (DTPA), showed no tendency to bind to serum proteins under circumstances in which the ^{99m}Tc-SHNH-labeled oligonucleotide was largely protein bound (Hnatowich D.J., Winnard P. Jr., Virzi F, Fogarasi M, Sano T, Smith CL, Cantor CR, Rusckowski M., *J. Nucl. Med.*, 36: 2306-2314, 1995). This nonspecific protein binding can be attributed to use of the SHNH chelator for coupling the nucleic acid to ^{99m}Tc.

Summary of the Invention

The present invention relates to chelators for radionuclides, to compositions comprising the subject chelators, to methods for synthesizing the chelators and compositions, and to methods of use thereof.

In one aspect, the present invention provides novel chelator compositions for conjugation to polymers and targeting agents, to enable complexation of radionuclides to these macromolecules, for targeting agents for medical use. Chelators are used to bind a radionuclide to other molecular entities, including to such polymers as proteins and nucleic acids, which function as affinity ligands to medically important targets such as tumors and infectious disease organisms and infected cells. Further, these molecular entities are useful as conjugated affinity ligands to deliver a chelated radionuclide for pre-targeting drug strategies, to medical targets such as tumors and infectious organisms and infected cells, directing the radionuclide complex to a previously pre-targeted site.

In one embodiment, the invention provides compositions comprising a polymer, for example, a nucleic acid covalently linked to an oligopeptide tetradentate chelator moiety, in which a radionuclide is complexed with the chelator moiety. In a preferred embodiment, the polymer is a nucleic acid. The nucleic acid is preferably selected from the group consisting of deoxyribonucleic acid, ribonucleic acid, phosphorothioate nucleic acid, and peptide nucleic acid. In one embodiment, the nucleic acid directs the complex to a target, by virtue of that portion of the nucleic acid sequence of the complex that is complementary to a nucleic acid sequence specifically expressed by tumor cells.

In certain embodiments, the covalent bond by which the nucleic acid is conjugated to the chelator moiety is through a nitrogen atom of the nucleic acid. In preferred embodiments the chelator moiety is attached to the polymer through a group that is an ester, an amide, or a thioester. In preferred embodiments the bond between the chelator moiety and the polymer is not a thiourea. In preferred embodiments the

chelator moiety and the polymer are not linked through an aromatic residue. In preferred embodiments, the chelator moiety and the polymer are not linked through an amino acid side chain. The nitrogen atom can be linked to a terminal nucleotide of the nucleic acid, e.g., by using a linker. In preferred embodiments, the chelator moiety comprises at least two peptide residues. In preferred embodiments, the chelator moiety comprises at least one sulfur atom.

Preferred radionuclides are medically useful for imaging and for therapeutic applications, and are chosen on the basis of the energy spectrum of emission and the half-life of the radionuclide. Preferred radionuclides include technetium-93m, technetium-95m, technetium-99m, rhenium 186, rhenium 188 and rhenium 189. The most preferred radionuclide is technetium-99m. The invention provides pharmaceutical compositions comprising a nucleic acid covalently conjugated to an oligopeptide tetradentate chelator moiety and complexed to a radionuclide, in a pharmaceutically acceptable carrier. In preferred embodiments, the efficiency of labeling of the chelator-polymer with radionuclide is at least about 20%, 30%, 40%, 50%, 60%, 70%, or 80%.

In another aspect, the invention provides an activated ester of an acetyl-protected mercaptoacetyl oligopeptide chelator. In a preferred embodiment, the activated ester is an N-hydroxysuccinimide ester. In another embodiment, the invention provides an activated ester wherein the chelator is biotinylated.

In preferred embodiments, at least one residue of an oligopeptide chelator is selected from the group consisting of glycine, serine, threonine, proline, alanine, methionine, valine, isoleucine, tryptophan, tyrosine, and phenylalanine. In a preferred embodiment, the oligopeptide chelator is a lower alkyl-[N-[N-[N-mercaptoacetyl]]]-tripeptide, for example an acetyl-[N-[N-[N-mercaptoacetyl]]]-tripeptide. In a preferred embodiment, the chelator tripeptide is triglycine or triserine. In preferred embodiments, the tripeptide moiety does not contain an aromatic residue.

In another embodiment, the invention provides compositions in which a targeting moiety is covalently linked to a tetradentate mercaptoacetyl oligopeptide chelator moiety protected by a protecting group of the formula -C(O)-lower alkyl, more preferably, acetyl. In a preferred embodiment, the targeting moiety is a protein or polypeptide, which in a more preferred embodiment is an antibody, an antibody fragment, or a binding protein. In other preferred embodiments, the targeting moiety is a nucleic acid sequence. In certain preferred embodiments, the targeting moiety is avidin or streptavidin. In certain preferred embodiments, the targeting moiety is a growth factor.

In another preferred embodiment, compositions of chelator and targeting moiety comprise a nucleic acid covalently bound to the chelator. The nucleic acid is a DNA,

RNA, phosphorothioate, or a PNA polymer. The target molecule need not be a macromolecule, and in another preferred embodiment is a biotin compound. In preferred embodiments, the chelator moiety comprises a sulfur atom protected by a protecting group of the formula -C(O)-lower alkyl, more preferably, acetyl. A feature of this protecting group is the ability to remove it during chelation under mild conditions, enabling the practitioner of the invention to couple the targeting moiety-chelator composition to a radionuclide using without denaturation of heat-labile targeting moieties such as antibodies and antibody fragments. The radionuclidecan be complexed to the targeting moiety-chelator just prior to use. The invention provides pharmaceutical compositions of the targeting moiety-chelator composition in a pharmaceutically acceptable carrier.

In another aspect, the invention provides a method of synthesizing an activated ester of an S-protected mercaptoacetyl amino acid. The method includes steps of reacting an amino with an activated ester of an S-protected thioglycolic acid under conditions such that an S-protected mercaptoacetyl amino acid and an activating alcohol are formed, and reacting the S-protected mercaptoacetyl amino acid and the activating alcohol with a coupling reagent under conditions such that an activated ester of an S-protected mercaptoacetyl amino acid is formed. In a preferred embodiment, the amino acid is an oligopeptide. In preferred embodiments, the tripeptide moiety does not contain an aromatic residue. In a preferred embodiment, the coupling reagent is dicyclohexylcarbodiimide. In a preferred embodiment, additional activating alcohol is provided in the second step of the synthesis. The most preferred oligopeptide is a tripeptide, preferably a tripeptide comprising at least two residues of glycine or serine.

The invention provides a method of forming under mild conditions a polymer-chelator-radionuclide complex, comprising the steps of contacting the polymer-chelator composition with a radionuclide, the chelator being a -C(O)-lower alkyl-protected tetradentate mercaptooligopeptide, and allowing the polymer-chelator-radionuclide complex to form under mild conditions. In one preferred embodiment of this method, the polymer is a protein or a polypeptide, and in another preferred embodiment, the polymer is a nucleic acid. Most preferably, the radionuclide is technetium-99m, and the technetium-99m is provided in the form of a pertechnetate. The invention also features polymer-chelator-radionuclide complex formed by this method, and a pharmaceutical composition comprising such a polymer-chelator-radionuclide complex in a pharmaceutically acceptable carrier. In another aspect, the invention provides a method of treating an infectious disease, comprising the steps of: (a) administering to a subject a high specific activity preparation of the polymer-chelator-radionuclide composition of

the invention, in a pharmaceutically acceptable carrier; and (b) allowing the polymer-chelator-radionuclide composition to accumulate at the site of the infection and irradiate the infectious agents and the infected cells, such that the infectious disease is treated.

The invention includes also a method for detecting a tumor, comprising the steps of administering a polymer-chelator-radionuclide of the invention, to a subject with a tumor, allowing the radioactivity to accumulate at the tumor site, and detecting a radioactive signal to detect a tumor. In preferred embodiments, the tumor is detected by converting the radioactive signal to generate an image of a tumor. The invention also provides a method for treating a tumor, comprising the steps of administering to a subject in need thereof a high specific activity preparation of a polymer-chelator-radionuclide of the invention, and allowing the radio-labeled complex to accumulate at the tumor site and destroy the tumor cells.

In another embodiment, the invention provides a method for imaging of an infectious disease site, comprising the steps of administering to a subject a targeting moiety-chelator-radionuclide composition of the invention in a pharmaceutically acceptable carrier, the composition having affinity for a target on an infectious agent or an infected cell, and binding the composition to the infectious agent or infected cell. Another method for treating an infectious disease is provided, in which a subject receives a high specific activity preparation of a polymer-chelator-radionuclide composition of the invention in a pharmaceutically acceptable carrier. The polymer-chelator-radionuclide composition accumulates at the site of the infection, and irradiates the infectious agents and the infected cells, treating the infectious disease.

Another preferred method for therapy of a subject with a tumor comprises administering to the subject a pre-targeting composition with affinity for a tumor marker, allowing a complex of said tumor marker and pre-targeting agent to form, then administering a radiolabeled targeting agent, which is a composition comprising a polymer-chelator-radionuclide of the invention. The radio-labeled complex can accumulate at the site of the tumor and destroy the tumor cells.

In another embodiment, a method for therapy of a subject with an infection is provided, in which the subject receives a pre-targeting composition with affinity for an infectious organism or infected cell, and a complex is formed of the infectious organism or infected cell and the pre-targeting agent. Then the subject is administered a polymer-chelator-radionuclide composition of the invention. The radio-labeled complex accumulates at the site of the infection and destroys the infectious organisms or infected cells.

In another aspect, the invention provides kits comprising compositions of the invention. In a preferred embodiment, a kit comprises an acetyl-protected tetradentate oligopeptide chelator covalently linked to a targeting moiety (optionally including reagents for complexing said chelator with a radionuclide under mild conditions, and a pharmaceutically acceptable carrier), a container, and instructions for use. In another embodiment, a kit comprises a targeting moiety covalently linked to a tetradentate oligopeptide chelator moiety protected by a group represented by the formula -C(O)-lower alkyl, (optionally including reagents for complexing the chelator composition with a radionuclide, a pharmaceutically acceptable carrier) a container, and instructions for use. In a preferred embodiment, the kit further comprises a pre-targeting agent.

Brief Description of the Drawings

Figure 1 is a scheme showing a synthesis of acetyl-protected NHS-MAG_3.

Figure 2 depicts radiochromatograms obtained by size exclusion HPLC analysis. Panel A: of ^{99m}Tc-MAG_3-DNA; Panel B: after adding the radiolabeled DNA to biotin saturated avidin; Panel C: after adding the radiolabeled DNA to unsaturated avidin; Panel D: after adding the radiolabeled DNA to avidin to which the complementary DNA was previously bound.

Figure 3 shows radiochromatograms obtained by size exclusion HPLC analysis of SHNH-coupled DNA (left column) and MAG_3-coupled DNA (right panel) after labeling with ^{99m}Tc. Panel A: of the labeled DNAs themselves; Panel B: following incubation of the labeled DNAs in solution with a 650-fold molar excess of cysteine; Panel C: after 1 hr of incubation in 37°C serum.

Figure 4 shows radiochromatograms obtained by size exclusion HPLC analysis of ^{99m}Tc-MAG_3-DNA itself (top row) and after 10 min to 24 hr in 37°C serum.

Figure 5 shows size exclusion HPLC radiochromatograms of labeled PNA in buffer (A), after the addition of biotinylated complementary PNA (B), and after the addition of avidin to the PNA-PNA duplex (C).

Figure 6 shows the rate of hybridization of labeled PNA in pH 7 buffer to complementary PNA immobilized on beads. Control refers to identical study using beads without PNA.

Figure 7 shows size exclusion HPLC radiochromatograms of labeled PNA in buffer (A), after 1 (B) and 24 hrs (C) of incubation in 37°C human serum, and after 24 hrs in saline (D).

Figure 8 shows size exclusion HPLC radiochromatograms of labeled PNA in buffer (A), labeled PNA in 37°C human serum for 1.5 hr (B), the 1.5 hr serum sample

after removing labeled PNA by adding complementary PNA immobilized on beads (C), and the 1.5 hr serum sample after adding beads without complementary PNA as control.

Figure 9 shows size exclusion HPLC radiochromatograms of labeled PNA in buffer (A) and of several samples obtained at 2.5 hrs post administration of labeled PNA to a mouse which include a serum sample (B), the soluble fraction of a kidney homogenate (C), urine (D) and the 2.5 hrs urine sample (B), the 1.5 hr serum sample after adding complementary PNA immobilized on beads to extract radiolabeled PNA (C) and the 2.5 hrs urine sample after removing labeled PNA with complementary PNA immobilized on bead (E).

Figure 10 shows whole body radioactivity as a function of time (each of the experimental animals plotted as a different symbol) following injection of radiolabeled PNA.

Figure 11 shows the percentage of injected radioactivity in the left thigh (closed circles) and right thigh (open circles) as a function of time after administration of labeled PNA to mice implanted with complementary PNA-containing beads in the left thigh (left scale), and the left/right thigh radioactivity ratio as a function of time (inverted triangles, right scale).

Figure 12 shows stability of the chelated ^{99m}Tc to cysteine challenge for the antibodies Sandoz and C110, each antibody conjugated to radionuclide with each of the MAG_3 and SHNH chelators.

Figure 13 shows the synthesis of biocytin conjugates of N_3S chelators.

Figure 14 shows the structures of N_2S_2-1, -2 and -3 N_2S_2 chelators, and synthesis of N_2S_2-1 chelators and corresponding biocytin conjugates.

Figure 15 shows the synthesis of N_2S_2-2 and N_2S_2-3 NHS chelators and corresponding biocytin conjugates.

Detailed Description of the Invention

For convenience, certain terms employed in the specification, examples and appended claims are collected here.

The term "radionuclide", as used herein, refers to a radioactive isotope of a selected element, preferrably a metallic element. Preferred radionuclides include isotopes of technetium and rhenium, and more preferably the isotopes technetium-93m, technetium-95m, technetium-99m, rhenium 186, rhenium 188 or rhenium 189. In a most preferred embodiment, the radionuclide is technetium-99m, particularly in the form of $Tc(O)^{3+}$. Technetium-99m has a half-life of 6.0 hours and an energy of decay of 0.14

MeV (Handbook of Chemistry and Physics, CRC Press, 64th edition, 1983), and has been widely used for *in vivo* and *in vitro* labeling studies and diagnostic applications.

The term "subject," as used herein, refers to a living animal or human in need of diagnosis or prognosis for, or susceptible to, a condition, in particular an infectious disease or a cancer as defined below. In preferred embodiments, the subject is a mammal, including humans and non-human mammals such as dogs, cats, pigs, cows, sheep, goats, horses, rats, and mice. In the most preferred embodiment, the subject is a human. The term "subject" does not preclude individuals that are entirely normal with respect to infections and cancer or normal in all respects.

The term "patient," as used herein, refers to a human subject who has presented at a clinical setting with a particular symptom or symptoms suggesting one or more diagnoses or the need for an imaging procedure. A patient may be in need of further categorization by clinical procedures well-known to medical practitioners of the art (or may have no further disease indications and appear to be in any or all respects normal). A patient's diagnosis may alter during the course of disease progression, such as development of further disease symptoms, or remission of the disease, either spontaneously or during the course of a therapeutic regimen or treatment. Thus, the term "diagnosis" does not preclude different earlier or later diagnoses for any particular patient or subject.

As used herein, the term "nucleic acid molecule" is intended to include naturally occurring DNA molecules (e.g., cDNA or genomic DNA), RNA molecules (e.g., mRNA), synthetic oligomer and poly-deoxyribonucleotides and ribonucleotides, and analogs of DNA, such as peptide nucleic acid molecules (PNA), phosphorothioate DNA, and DNA based on a peptide analog backbone such as trans-olefin peptidomimetics and phosphonate peptidomimetics. Peptide nucleic acid (PNA) is an oligomer in which the charged phosphate-ribose backbone has been eliminated and replaced with an uncharged polyamide backbone (Egholm M., Buchardt O., Nielsen PE., Berg RH., *J. Am. Chem. Soc.*, 114: 1895-1897; 1992). These oligomers have been reported to resist nuclease and protease degradation (Egholm M., Buchardt O., Christensen L., Behrens C., Freier SM., Driver DA., Berg RH., Kim SK., Norden B., Nielsen PE., *Nature*, 365: 566-568; 1993.). Furthermore, the binding affinities of PNA for its complementary single-stranded PNA has been shown to exceed that of comparable DNAs (Egholm M., Buchardt O., Nielsen PE., Berg RH., *J. Am. Chem. Soc.*, 114: 1895-1897; 1992).

The invention further contemplates the use of nucleic acids, polynucleotides, and oligonucleotides that are alternatives to, or analogs of, naturally occuring deoxynucleic acid with its sugar-phosphate backbone, or synthetic oligo-and polynucleotide sugar-

phosphate polymers. While native single-stranded phosphodiester DNA has been considered for *in vitro* or *in vivo* applications; these unmodified oligonucleotides are highly susceptible to degradation by nucleases (Wickstrom E. *J Biochem Biophys Methods*. 13:97-102, 1986; Cazenave C., Chevrier M., Ngugent T, Helene C. *Nucleic Acids Res.* 15:10507-10521, 1987; Ceruzzi M. Draper K. *Nucleosides Nucleotides* 8: 815-818, 1989) and, as such may be inappropriate as carrier of radioactivity for certain *in vivo* imaging, diagnostic and therapeutic applications. Methods have been developed to chemically modify the phosphodiester DNA to improve its stability (Goodchild J. *Bioconj. Chem.* 1: 165-187, 1990). Among the many possible modified DNAs, phosphorothioates, in which a nonbonding oxygen in the phosphate backbone is replaced with a sulfur (Iversen P. In *Antisense research and applications*, Crooke ST, Lebleu B. eds. CRC Press, Ann Arbor MI, 1993 p462-469), have found considerable application. Data from investigations of a phosphorothioate DNA radiolabeled with ^{99m}Tc indicate, however, that there can be a high affinity for serum and tissue proteins and resulting unfavorable pharmacokinetics (Hnatowich DJ, Mardirossian G, Fogarasi M, Sano T, Smith CL, Cantor CR, Rusckowski M, Winnard P. Jr.. *Pharm Exp Therap* 276: 326-334; 1996.). The native phosphodiester DNA and RNA, and phosphorothioate nucleic acids are included in the present invention. as their properties may be superior for some applications.

A nucleic acid molecule may be single-stranded or double-stranded, but preferably is single-stranded. In preferred embodiments, the nucleic acid is DNA, PNA phosphorothioate DNA, or RNA, and most preferably is PNA.

As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence and structure of backbone that occurs in nature, whether it is prepared by isolation from an organism or is chemically synthesized. A nucleic acid may be chemically synthesized using a commercially available automated synthesizer and reagents, or custom made by a commercial supplier (for example, PerSeptive Biosystems, Framingham, MA). A "chimeric nucleic acid" is a covalently linked first base sequence with a second base sequence of different chemical character, for example, a PNA strand covalently linked to a DNA or RNA strand. An "isolated" nucleic acid molecule is free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, an isolated tumor-specific nucleic acid molecule may contain less than about 5 kb, 1 kb, 0.5 kb, 0.1 kb or 50 bases of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid

is derived (e.g. a human brain tumor). Moreover, an "isolated" nucleic acid molecule, such as an RNA molecule, may be free of other cellular material. The nucleic acid molecule may comprise only a portion of a coding region of a naturally occurring sequence.

In a preferred embodiment, an isolated nucleic acid molecule of the invention comprises a base nucleotide sequence with a specific linear order of the bases adenine, guanine, cystosine, thymine or uracil, or modified derivatives of these bases (e.g., methyladenine, and hydroxymethyluracil). A nucleic acid can contain non-naturally occurring bases, such as 5-fluorouracil, or "unusual" bases, such as ribothymidine or others, e.g., such as are found in tRNA. Other modified bases are known to those with skill in the art. The term "unusual base," is known in the art, and refers to uncommon bases such as pseudouracil or ribothymine, found, e.g., in tRNA. It is also possible to modify the structure of the oligonucleotides and polynucleotides for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., *ex vivo* shelf life and resistance to nucleolytic degradation *in vivo*).

A nucleic acid molecule having a known nucleotide sequence can be isolated using standard molecular biology techniques (e.g., as described in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd. ed.*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). For example, mRNA can be isolated from cells (e.g., by the guanidinium-thiocyanate extraction procedure of Chirgwin *et al.* (1979) *Biochemistry* 18: 5294-5299) and cDNA can be prepared using reverse transcriptase (e.g., Moloney MLV reverse transcriptase, available from Gibco/BRL, Bethesda, MD; or AMV reverse transcriptase, available from Seikagaku America, Inc., St. Petersburg, FL). Synthetic oligonucleotide primers for PCR amplification can be designed based upon knowledge of the nucleotide sequence or fragments of the sequence, as will be appreciated by those with skill in the art, and are used to obtain and clone an isolated nucleic acid adjacent to the primer.

In certain embodiments, an isolated nucleic acid molecule useful in the compositions and methods of the invention is at least about 12 nucleotides in length and hybridizes under stringent conditions to a nucleic acid molecule comprising the nucleotide sequence of interest. In other embodiments, a nucleic acid is at least 15, 20, 30, 50, or 100 nucleotides in length. As used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences at least 60 % homologous to each other typically remain hybridized to each other. Preferably, the conditions are such that at least sequences at least 65 %, more preferably at least 70 %, and even more preferably at least

75 % homologous to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found, e.g.,in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. A preferred, non-limiting example of stringent hybridization conditions is hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50-65°C. Preferably, an isolated nucleic acid molecule of the invention that hybridizes under stringent conditions to a sequence of the invention corresponds to a naturally-occurring nucleic acid molecule. A "portion" of a nucleic acid sequence refers to a subsequence of a nucleic acid sequence. The "portion" can be an entire nulceic acid sequence or any subsequence thereof. In preferred embodiments, a portion is at least about 6 nucleotide bases in length, more preferably at least about 12 bases in length, ansstill more preferably at least about 15 bases in length.

The term "can be degraded *in vivo*", as used herein, refers to a polymer that includes a bond that can be cleaved *in vivo*, either enzymatically or non-enzymatically. For example, natural nucleic acids can be cleaved by nucleases that attack the sugar-phosphate backbone, at any phosphodiester bond or at specific sites of defined base sequence.

In addition to nucleic acid molecules with sequences of interest, the invention contemplates use of nucleic acid molecules which are antisense thereto. An "antisense" nucleic acid comprises a nucleotide sequence which is complementary to a "sense" nucleic acid, e.g., complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Antisense constructs of the present invention, by antagonizing the normal biological activity of tumor-specific or infectious DNA, can be used in the therapeutic context, both to deliver a therapeutic dose of radionuclide, and to inhibit expression of tumor-specific or infectious genetic information. In a preferred embodiment, antisense nucleic acid can deliver a radionuclide complexed to a chelator covalently linked to the nucleic acid, to the target cells, tissue or sample.

The skilled artisan will appreciate that antisense nucleic acids can be designed according to methods known in the art, e.g., according to the rules of Watson and Crick base pairing. The antisense nucleic acid can be complementary to an entire coding strand, or to only a portion thereof. In one embodiment, an antisense nucleic acid molecule is antisense to a "coding region" of the coding strand of a nucleotide sequence. The term "coding region" refers to the region of the nucleotide sequence comprising codons which are translated into amino acid residues. In another embodiment, the antisense nucleic acid molecule is antisense to a "noncoding region" of the coding strand

of a nucleotide sequence of interest. The term "noncoding region" refers to 5' and 3' sequences which flank a coding region and are not translated into amino acids (i.e., also referred to as 5' and 3' untranslated regions). Antisense nucleic acid may be complementary to either or both of a coding region and an adjacent noncoding region.

The antisense nucleic acid molecule may be complementary to an entire coding region, but more preferably is an oligonucleotide which is antisense to only a portion of a coding or noncoding region. For example, an antisense oligonucleotide may be complementary to the region surrounding the translation start site of an mRNA. An antisense oligonucleotide can be, for example, about 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An antisense nucleic acid of the invention can be constructed using chemical synthesis and/or enzymatic reactions using procedures known in the art. For example, an antisense nucleic acid (e.g., an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, e.g., peptide nucleic acid or phosphorothioate nucleotides can be used. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e., RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid).

Nucleic acids of the present invention are suitable molecules for the purposes of therapeutic "pretargeting," in which administration of a first composition, e.g., a pretargeting agent, to a subject causes the administered composition, agent, or material to become located or concentrated at a particular, preferably preselected, *in vivo* site. A second composition, e.g., a targeting agent, is subsequently administered which, by virtue of ligand affinity for the first-administered material, is specifically targeted to the *in vivo* site. The molecular affinity of the pretargeted material for the *in vivo* site is, in a preferred embodiment, achieved by use of a specific antibody or antibody fragment, such as an antibody to a tumor marker. In certain embodiments, a pretargeting agent can be a binding protein with engineered ligands, or an antisense nucleic acid of specific nucleotide sequence.

A second binding determinant of a pretargeting agent is a ligand with affinity for the subsequently administered "targeting" complex, agent or composition. In an illustrative embodiment, a pretargeting agent or complex carries a nucleic acid sequence, and the targeting agent or complex carries a nucleic acid sequence complementary to the sequence of the pretargeting agent. In another preferred embodiment, the pretargeting

complex includes avidin or streptavidin, and the targeting complex includes a biotin compound. Alternatively, the pretargeting complex can include a biotin compound, and the secondarily administered targeting complex includes avidin or streptavidin. It will be understood that other members of specific binding pairs, e.g., hormone/receptor, antibody/antigen, and the like, can be used as complementary pairs in targeting and pretargeting agents.

The pretargeting molecule or complex can facilitate the specific localization of the subsequently-administered targeting agent, which includes a functional moiety (e.g., a radionuclide) for therapeutic, diagnostic or imaging use. The advantage of pretargeting includes a greater localization ratio of specific to non-specific background of the targeted radionuclide for *in vivo* imaging and for therapy, leading to the use of lower doses of radioactive material, with enhanced contrast in imaging and fewer side effects in therapy. Further improvement in targeting specificity is achieved by addition of "chase" regimens, e.g., by administration of compositions that can displace non-specifically bound pretargeting complexes, thereby enhancing image contrast or therapeutic index following delivery of the targeting radionuclide composition.

A "targeting" composition may in certain embodiments be employed alone or in combination with any of variety of different pretargeting compositions. For example, several different pretargeting agents can be designed to have affinity for a certain specific type of targeted tumor or an infectious agent or infected cell, e.g., the pretargeting agents can be antibodies for different tumor-specific antigens of a tumor cell or cells. All of these pretargeting agents can share a common ligand, e.g., a specific nucleic acid sequence, for a targeting agent. Thus a variety of different tumor types and infectious diseases may be pretargeted, each with a specific pretargeting composition, then treated with the same general targeting composition, comprising for example, a complementary nucleic acidconjugated chelator which is complexed to a radionuclide. Thus, a particular targeting agent of the invention can be useful in several medical regimens, and a single targeting agent can be used for a variety of conditions by use of appropriate pretargeting agents.

As used herein, the term "polymer" is intended to include molecules formed by the chemical union of two or more combining subunits called monomers. Monomers are molecules or compounds which usually contain carbon and are of relatively low molecular weight and simple structure. A monomer can be converted to an oligomer or to a polymer by combination with itself or other similar molecules or compounds. An oligomer comprises at least two monomers. An oligomer or a polymer may be composed of a single identical repeating subunit or multiple different repeating subunits

(copolymers). Polymers within the scope of this invention include substituted and unsubstituted oligopeptides, carbohydrates, polypeptides, oligonucleotides, polynucleotides, and polypeptide backbones substituted with purine and purimidine bases or base analogs (e.g. PNA).

While reference is made herein to compositions in which a chelator moiety is conjugated to a polymer or macromolecule, the skilled artisan will appreciate that the chelator moieties of the invention can be conjugated to any suitably functionalized molecule. Thus, a chelator moiety can be conjugated to small molecules, such as drugs, provided that the small molecule includes suitable reactive functionality, e.g., an amino group, for reaction with the chelator moiety.

The term "peptide" includes two or more amino acids covalently attached through a peptide bond. Amino acids which can be used in peptide molecules include those naturally occurring amino acids found in proteins such as glycine, alanine, valine, cysteine, leucine, isoleucine, serine, threonine, methionine, glutamic acid, aspartic acid, glutamine, asparagine, lysine, arginine, proline, histidine, phenylalanine, tyrosine, and tryptophan. In preferred embodiments, the tripeptide moiety does not contain an aromatic residue. The term amino acid further includes analogs, derivatives and congeners of naturally occurring or synthetic amino acids, one or more of which can be present in a peptide derivative. For example, amino acid analogs can have lengthened or shortened side chains or variant side chains with appropriate functional groups. The present invention does not require modification of the side chain of the amino acids of the chelator. Also included are the D and L stereoisomers of an amino acid when the structure of the amino acid admits of stereoisomeric forms. The term "peptide derivative" further includes compounds which contain molecules which mimic a peptide backbone but are not amino acids (so-called peptidomimetics), such as benzodiazepine molecules (see e.g. James, G. L. *et al.* (1993) *Science* 260:1937-1942). An oligopeptide can be designed to interact with a cell membrane constituent (e.g., if comprised primarily of hydrophobic amino acids). Accordingly, in one embodiment, an oligopeptide comprises three or four peptide residues, and a polypeptide comprises four or more residues. Polymers comprising oligopeptides or peptide backbones may be covalently linked to other moieties or functionalitites, for example, to an amine group attached via a linking arm.

The term "alkyl", as used herein, refers to a straight or branched chain hydrocarbon group having from about 1 to about 20 carbon atoms. The term "lower alkyl" refers to an alkyl group having from 1 to 6 carbon atoms. Exemplary lower alkyl groups include methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl.

n-pentyl, and n-hexyl. A C_1-C_3 alkyl refers to an alkyl group having 1 to 3 carbon atoms. An alkyl group may be unsubstituted, or may be substituted at one or more positions. It will be understood by those skilled in the art that the moieties substituted on the hydrocarbon chain can themselves be substituted, if appropriate. For instance, the substituents of a substituted alkyl may include substituted and unsubstituted forms of aminos, azidos, iminos, amidos, phosphoryls (including phosphonates and phosphinates), sulfonyls (including sulfates, sulfonamidos, sulfamoyls and sulfonates), and silyl groups, as well as ethers, alkylthios, carbonyls (including ketones, aldehydes, carboxylates, and esters), -CF_3, -CN and the like. Preferred alkyls are lower alkyls.

The term "aryl" as used herein, refers to an aromatic ring moiety having zero to four heteroatoms, and which may be substituted or unsubstituted, and can be fused to other aromatic or non-aromatic rings. Exemplary aryls are phenyl, pyridyl, naphthyl, and the like. The term "aralkyl" refers to an alkyl group substituted with one or more aryl moieties, e.g., benzyl.

The term "antibody" as used herein is intended to include fragments thereof which are also specifically reactive with one of the imaging, diagnostic and therapeutic targets. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, $F(ab)_2$ fragments can be generated by treating an antibody with pepsin. The resulting $F(ab)_2$ fragment can be treated to reduce disulfide bridges to produce Fab fragments. The term "antibody" is further intended to include single chain, bispecific and chimeric molecules. The term "antibody" includes possible use both of monoclonal and polyclonal antibodies (Ab) directed against a target, according to the requirements of the application.

Polyclonal antibodies can be obtained by immunizing animals, for example rabbits or goats, with a purified form of the antigen of interest, or a fragment of the antigen containing at least one antigenic site. Conditions for obtaining optimal immunization of the animal, such as use of a particular immunization schedule, and using adjuvants e.g. Freund's adjuvant, or immunigenic substituents covalently attached to the antigen, e.g. keyhole limpet hemocyanin, to enhance the yield of antibody titers in serum, are well-known to those in the art,. Monoclonal antibodies are prepared by procedures well-known to the skilled artisan, involving obtaining clones of antibody-producing lymphocyte, i.e. cell lines derived from single cell line isolates, from an animal, e.g. a mouse, immunized with an antigen or antigen fragment containing a minimal number of antigenic determinants, and fusing said clone with a myeloma cell line to produce an immortalized high-yielding cell line. Many monoclonal and

polyclonal antibody preparations are commercially available, and commercial service companies that offer expertise in purifying antigens, immunizing animals, maintaining and bleeding the animals, purifying sera and IgG fractions, or for selecting and fusing monoclonal antibody producing cell lines, are available.

Specific binding proteins with high affinities for targets can be made according to methods known to those in the art. For example, proteins that bind specific DNA sequences may be engineered (Ladner *et al.*, U.S. Patent 5,096,815), and proteins that bind a variety of other targets, especially protein targets (Ladner *et al.*, U.S. Patent 5,233,409; Ladner *et al.*, U.S. Patent 5,403,484) may be engineered and used in the present invention for covalent linkage to a chelator moiety. Antibodies and binding proteins can be incorporated into large scale diagnostic or assay protocols that require immobilizing the compositions of the present invention onto surfaces, for example in multi-well plate assays, or on beads for column purifications.

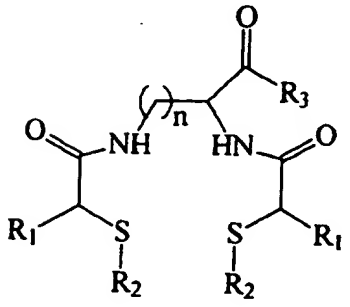
The term "chelator", as used herein, refers to a moiety that is capable of binding a radionuclide, preferably through non-covalent interactions, e.g., through ionic interactions. The term "chelator moiety" is also used herein to describe S-protected forms of moieties which, in an unprotected state, are capable of chelating a radionuclide, although the skilled artisan will recognize that such a protected moiety may not be capable of chelation until the protecting group is removed. Thus, for example, an S-acetyl mercaptoacetyltripeptide is sometimes referred to herein as a chelator moiety. Chelator moieties suitable for use in the compositions and methods of the invention are preferably capable of binding to a radionuclide with a high affinity, e.g., a binding affinity sufficiently high to permit binding of a radionuclide, preferably under physiological conditions, e.g., *in vivo*. It will be understood that a chelator moiety which comprises peptide residues, e.g., an oligopeptide chelator, can be derivatized, e.g., with a mercaptoacetyl moiety. The term "nucleic acid-chelator" refers to a compound comprising a nucleic acid, including PNA, covalently bound to a chelator moiety. Similarly, "protein-chelator" refers to a protein, including an antibody, covalently bound to a chelator moiety.

Chelators which bind to radionuclides are known in the art, see, e.g., M. Nicolini *et al.*, eds., "Technetium and Rhenium in Chemistry and Nuclear Medicine," SGEditoriali, Padova (1995). In general, preferred chelators are capable of binding to radionuclides such as $Tc(O)^{3+}$. In a preferred embodiment, a chelator moiety will be a tetradentate chelator, i.e., will be capable of four-point binding to a radionuclide. Exemplary tetradentate chelators include N_2S_2 and N_3S chelators, as described in, e.g., A.R. Fritzberg, *et al.*, *J. Nucl. Med.* 23:592-598 (1982); S. Liu and D.S. Edwards, *in* M.

Nicolini *et al.*, eds., "Technetium and Rhenium in Chemistry and Nuclear Medicine," *op. cit.*, pp. 383-393; and S. Vallabhajousula *et al.*, *J. Nucl. Med.* 30:599-604 (1989). An N_2S_2 chelator can chelate a radionuclide through two nitrogen atoms (e.g., amido nitrogens, e.g., of a peptide backbone) and two sulfur atoms (e.g., of a mercaptoacetyl moiety), while N_3S chelators can chelate to a radionuclide through three nitrogen atoms and one sulfur atom.

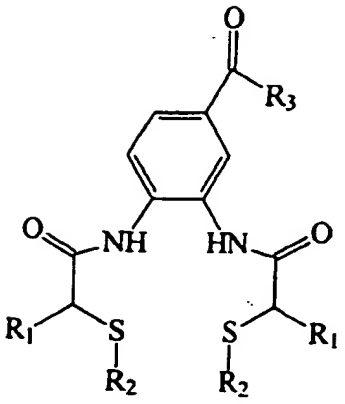
Accordingly, preferred chelator moieties include amidothiols, including, e.g., mercaptoacetyloligopeptides, and more preferably, mercaptoacetyltripeptides, such as, e.g., mercaptoacetyltriglycine (MAG_3), mercaptoacetyltriserine, and the like. Mercaptoacetyl-tripeptides can chelate radionuclides such as $Tc(O)^{3+}$ by coordination through the three amide nitrogens of the peptide backbone, and the terminal mercapto group. Other chelator moieties which may find use in the present invention include cyclams, porphyrins, crown ethers, azacrown ethers, and the like. As the skilled artisan will understand from the teachings herein, a chelator moiety will preferably be capable of covalently bonding to a polymer, e.g., a protein or a nucleic acid, e.g., DNA, RNA, phosphorothioate, or PNA, or other polymer compound. In preferred embodiments, the tripeptide moiety does not contain an aromatic residue. Thus, a mercaptoacetyltripeptide molecule can form an amide bond, e.g., through the C-terminal carboxyl moiety of the tripeptide, with a nitrogen atom of a peptide or a polymer (which can be derivatized if necessary to provide a suitable reactive moiety). Similarly, a mercaptoacetyltripeptide can form an ester bond to a polymer through an oxygen atom of the polymer. The chelator moiety can be covalently linked to the polymer through covalent bonds to other functionalities of the chelator moiety. For example, a mercaptoacetyltripeptide which includes an aspartate residue can form an ester or amide bond to a polymer through the side-chain carboxylate of the aspartate residue.

In preferred embodiments, an N_2S_2 chelator moiety can be represented by one of the following formulas:



I

or



II

in which n is 1 or 2; R_1 is, independently for each occurrence, selected from the group consisting of alkyl, cycloalkyl, aryl, aralkyl; and can be substituted or unsubstituted; R_2 is, independently for each occurrence, hydrogen or a protecting group; and R_3 is -OH, or with the carbonyl moiety to which it is attached, forms an active ester or a linking group; or a salt or ester thereof. In a preferred embodiment, the chelator moiety can be represented by formula I, and n is 1. In a preferred embodiment, the chelator moiety can be represented by formula I, and n is 2. In another preferred embodiment, the chelator moiety can be represented by formula II. In a preferred embodiment, R_1 is hydrogen. In preferred embodiments, R_2 is -C(O)-lower alkyl, more preferably acetyl. In certain preferred embodiments, R_3 is -OH. In other preferred embodiments, R_3 is selected such that an active ester is formed; e.g., R_3 is -N-hydroxysuccinimidyl. R_3 can also be a functionality linking the chelator moiety to a polymer; for example, where the chelator moiety is covalently linked to a polymer through an amide linkage, R_3 can be -N-polymer.

The term "protecting group" is known in the art and refers to a moiety which blocks reaction at a particular atom or reactive center. A "protected" compound is a compound in which at least one atom is blocked by a protecing group. Thus, an "S-protected" compound includes a sulfur atom that is blocked with a protecting group.

The term "activated ester", as used herein, refers to a derivative of a carboxylate moiety that is suitable for use in a coupling reaction with a nucleophile, e.g., to produce an ester or amide bond. A variety of activated esters are known in the art, see, e.g., G.A. Grant, Ed., "Synthetic Peptides: A User's Guide", W.H. Freeman, New York (1992), Chp.3, and M. Bodansky, "Principles of Peptide Synthesis", 2nd ed., Spring-Verlag

(1993). In general, an activated ester suitable for use in the present invention will be sufficiently stable to permit storage of the ester for a suitable period of time before coupling to a nucleic acid. Exemplary activated esters include, in addition to carboxylic esters, thioesters, acyl azides, anhydrides, mixed anhydrides, and the like. A preferred activated ester is an N-hydroxysuccinimide (NHS) ester, which is readily available from inexpensive starting reagents, see, e.g., Example 2, *infra*. Activated esters of the invention do not include isothiocyanate derivatives.

The term "activating compound," as used herein, refers to a compound which, when esterified with a carboxylate, forms an activated ester. Similarly, the term "activating alcohol," as used herein, refers to an alcohol or hydroxyl-containing compound which, when esterified with a carboxylate, forms an activated ester. Thus, activating compounds include activating alcohols, thiols, acid halides, and the like. Exemplary activated alcohols include N-hydroxysuccinimide, pentafluorophenol, HOBt, and the like. N-hydroxysuccinimide is particularly preferred.

The term "coupling reagent" as used herein, refers to a reagent capable of effecting or promoting coupling of, e.g., an amine and a carboxylate, to form an amide, or an alcohol and a carboxylate to form an ester. Coupling reagents are known in the art, see, e.g., M. Bodansky, "Principles of Peptide Synthesis", 2nd ed., *supra*. A preferred coupling reagent is dicyclohexylcarbodiimide (DCC).

As used herein, the term "mild conditions" refers to reaction conditions that do not result in significant degradation or destruction of a nucleic acid-chelator-radionuclide or protein-chelator-radionuclide composition. Thus, mild conditions generally include reaction in aqueous or aqueous-organic solution, at a pH range of about 5.5 to about 8.5, more preferably about 6 to about 8. Mild conditions also generally feature temperatures less than 100° C, more preferably less than 80° C, and still more preferably less than 60° C. Mild conditions include the absence of harsh reagents or high salt concentrations that would cause significant loss of structural integrity or secondary and tertiary structural features of polymeric molecules such as proteins. Conditions appropriate for a particular reaction will be dependent on the nature of the composition and the reagents. For example, an antibody-chelator composition may be unstable above a temperature of about 45° C due to denaturation of the antibody portion. Thus, mild conditions for radiolabeling an antibody-chelator composition may include a temperature no greater than 45° C, more preferably less than 40° C.

The rate or efficiency of conjugation can be affected by changes in reaction conditions such as temperature or pH. For example, elevated temperature can result in more rapid conjugation of radionuclide to the chelator; however it will be appreciated by

one of ordinary skill in the art that elevated temperatures can cause denaturation or degradation of certain polymers, such as proteins, and use of elevated temperature can be appropriate when use of such temperature will not denature or degrade the polymer. Similarly, polymers can be sensitive to extremes of pH. In certain embodiments, it is preferred that the temperature for conjugation and/or radionuclide complexation is not less than about 20° C, 30° C, 40° C, 50 ° C, 60 ° C, 70 ° C, or 80° C. In certain embodiments, the temperature is not greater than about 80 ° C, 70 ° C, 60 ° C, 50 ° C, or 40 ° C. In certain embodiments, the pH is in the range of pH 5.5 to 8.5.

As used herein, the term "biotin compound" refers to biotin, iminobiotin, biocytin, or derivatives, conjugates, or analogs thereof, which can bind specifically to avidin or streptavidin. Thus, for example, a biotinylated compound may be referred to herein as a "biotin compound." Biotin compounds are commercially available or can be synthesized by methods known in the art (see *infra*).

I. Compositions

In one aspect, the invention provides a composition comprising a polymer, e.g., a nucleic acid or peptide, in which the polymer is covalently linked to a chelator moiety, and a radionuclide is bound to the chelator. Such compositions are useful for, e.g., diagnosis or imaging or treatment of, e.g., cancer or infectious diseases, as is described in more detail *infra*.

In general, the DNA, phosphorothioate, peptide nucleic acid or ribonucleic acid will be selected to be complementary to a sequence of interest, e.g., a diagnostic sequence or a sequence on a pretargeting agent. While the nucleic acid need not be perfectly complementary to the sequence of interest, in preferred embodiments, complementarity will be sufficient to permit hybridization to the sequence of interest, either *in vitro* or *in vivo*, while substantially excluding non-specific hybridization to other sequences. In preferred embodiments, the nucleic acid is perfectly complementary to a sequence of interest.

The chelator moiety can be linked to the nucleic acid moiety directly, e.g., through an atom of the nucleic acid moiety, or through a linking moiety. For example, a chelator can be linked to the nucleic acid moiety through an atom of the backbone, e.g., a terminal amine, e.g., of a PNA, modified DNA or RNA, or protein; or through an oxygen atom, e.g., of a 3'- or 5'-terminal hydroxyl group, of , e.g.. an RNA. In certain embodiments, the nucleic acid moiety is modified, e.g., by addition of an amine group, to facilitate attachment of the chelator moiety. Thus, for example, DNA or RNA which has been chemically modified to have a terminal amine group (using linkers which can

be synthesized, or purchased from commercial sources, e.g., Operon Technologies, Alameda, CA) can be used to provide compositions of the invention. Alternatively, a chelator moiety can be linked to a nucleic acid base, e.g., a purine, a pyrimidine or a modified base, of a nucleic acid moiety. In general, it is preferred to attach the chelator moiety to a nucleic acid sugar moiety so as to substantially preserve the ability of the nucleic acid to bind to its complementary strand. In a preferred embosiment, attachment is at a terminal residue of the nucleic acid moiety. Linking moieties can be selected to permit the covalent attachment of the nucleic acid moiety to the chelator moiety without steric hindrance between the two moieties, while not significantly disturbing the ability of the nucleic acid strand to bind its complement. Suitable linking moieties are known in the art and selection of a linking moiety will be routine to the skilled artisan.

In a preferred embodiment, the ratio of chelator moiety to nucleic acid moiety is 1:1, i.e., there is one chelator moiety bound to each oligonucleotide or oligopeptide nucleic acid moiety. However, in certain embodiments, it may be preferred to attach more than one chelator moiety to a nucleic acid moiety. For example, higher levels of radioactivity can be achieved by chelating several radionuclide moieties to each nucleic acid moiety through a plurality of chelating moieties. Also, several different radionuclides can be associated with one nucleic acid chain by using a plurality of chelating moieties per nucleic acid; thus, several therapeutic or diagnostic radionuclides can be employed with a single nucleic acid moiety. In compositions that include a plurality of chelator moieties, the chelator moieties can all be the same or can be different.

The nucleic acid-chelator-radionuclide compositions of the invention can further comprise a group capable of specific bonding to a binding partner complement, which in a most preferred embodiment comprises the complementary strand of nucleic acid. Other binding partners contemplated for use in the instant invention include biotin and biotin compounds, including biotin derivatives and analogs; ligands for the biotin compounds including avidin or streptavidin; an antigen and an antibody; a receptor and its ligand; an engineered binding protein and its target, and the like. A nucleic acid-chelator-radionuclide composition can be bound, e.g., to a solid support, by contacting another binding moiety, e.g., through the complementary strand of nucleic acid. Alternatively, a biotin binding group or a biotin compound such as biocytin conjugated to the chelator complex can be bound by avidin or streptavidin, and any of these entities can be bound to a solid surface, e.g., a bead or a 96-well plate. Biotinylated polymers can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and the

compositions can be immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Exemplary methods for detecting such complexes include enzyme-linked assays, which rely on detecting an enzymatic activity associated with an enzyme activity linked to streptavidin or bound to streptavidin via an antibody. Illustratively, a polypeptide can be chemically cross-linked or genetically fused with alkaline phosphatase, and the amount of bound polypeptide trapped in the complex can be assessed with a chromogenic substrate of the enzyme, e.g. paranitrophenylphosphate. The addition of a binding group permits the ready isolation or purification of the nucleic acid complex from solution. Conveniently, biotinylated compounds can be readily immobilized for studying the *in vitro* binding or other properties of the nucleic acid-chelator or nucleic acid-chelator-radionuclide compositions of the invention. A chelator-protein composition can also be immobilized utilizing conjugation of biotin and streptavidin, or the like, and pretargeting and targeting compositions can be designed with high affinity by conjugation of one with a biotin compound and the other with avidin or streptavidin. Suitable methods and compositions will be apparent to the skilled artisan in light of the teachings herein.

The choice of polymer, e.g., nucleic acid (e.g., DNA, RNA, phosphorothioate or PNA) or protein, and chelator moiety will generally be guided according to at least some of the following criteria: 1) minimal non-specific binding *in vitro* or *in vivo* (e.g., little binding to serum proteins and the like); 2) ability to bind to specific nucleic acid sequences, e.g., diagnostic sequences, *in vitro* and/or *in vivo*; 3) stable complexation to a radionuclide; and 4) ease of synthesis. Thus, in preferred embodiments, a nucleic acid-chelator-radionuclide complex will bind to specific nucleic acid sequences, *in vivo* or *in vitro*, with relatively little non-specific binding, and the radionuclide will remain complexed under physiological conditions. The ability of a nucleic acid to bind to a complement, the extent of non-specific binding, and the binding affinity of the radionuclide for the chelator moiety can be assessed according to methods known in the art or described herein.

In another embodiment, the invention provides a composition comprising a nculeic acid (e.g., deoxyribonucleic acid, peptide nucleic acid, phosphorothioate or ribonucleic acid) and a chelator moiety covalently linked to the nucleic acid. Such compositions are useful, e.g., for synthesizing nucleic acid-chelator-radionuclide compositions such as are described herein.

In preferred embodiments, the chelator moiety is a mercaptoacetyl oligopeptide, more preferably a tripeptide, i.e., a tripeptide covalently linked (preferably at the amine terminus) to a mercaptoacetyl moiety, i.e., -C(O)-CH_2SR, wherein R is hydrogen or a

protecting group. In preferred embodiments, the mercapto group is protected, preferably as a lower alkyl thioester, e.g., -S-C(O)-lower alkyl, prior to chelation with the radionuclide. Thus, a preferred protecting group for the mercapto group can be represented by the formula -C(O)-lower alkyl; in more preferred embodiments, the lower alkyl is a C_1-C_3 alkyl, and in a most preferred embodiment, the lower alkyl is a methyl group (e.g., the protecting group is an acetyl group). The protecting group can prevent undesired side reactions at the sulfur atom, e.g., oxidation, during synthesis or storage of the nucleic acid-chelator compound. Protecting groups for sulfur are known (see, e.g., T. Greene and P. Wuts, "Protective Groups in Organic Synthesis," J. Wiley, (1991)). In preferred embodiments, the sulfur protecting group can be removed under mild conditions to unveil the free mercapto group, which can then participate in chelation of a radionuclide, e.g., $Tc(O)^{3+}$. In a particular advantage of the present invention, a sulfur protecting group can be removed under mild conditions to allow the chelation of a radionuclide to the nucleic acid-chelator compound concomitant with removal of the protecting group, reducing the number of synthetic steps required, reducing radioactive exposure of personnel, improving economy of radionuclide usage, and simplifying the experimental conditions. Importantly, the mild conditions enable use of the method with polymers such as proteins that would otherwise be denatured. Thus, for example, an S-acetyl protecting group can be removed under conditions used for reduction of pertechnetate to $Tc(O)^{3+}$, e.g., in the presence of $SnCl_2$ at near neutral pH and at room temperature (see Example 4, *infra*), simultaneously generating a chelatable form of the radionuclide and the chelator moiety, and permitting chelation to occur, for example, with a chelator-protein composition.

In another embodiment, the invention provides a composition comprising a peptide nucleic acid covalently linked to an amidothiol chelator moiety, e.g., a chelator moiety comprising at least one amido nitrogen and at least one sulfur which can chelate to a radionuclide. In preferred embodiments, the amidothiol chelator moiety is a tripeptide or oligopeptide derivatized with a sulfur-containing moiety, e.g., a mercaptoacetyl moiety, such that the amidothiol chelator moiety can complex a radionuclide, e.g., in a tetradentate complex. In a particularly preferred embodiment, the amidothiol chelator moiety is a mercaptoacetyltripeptide.

In another embodiment, the invention provides a kit comprising a polymer-chelator compound in a container, and instructions for complexing the polymer-chelator compound with a radionuclide. In preferred embodiments, the polymer-chelator compound is a nucleic acid-chelator compound. In preferred embodiments, the nucleic acid is complementary to a tumor-specific nucleic acid, and in other preferred

embodiments, the nucleic acid is complementary to nucleic acid of a pathogenic organism selected from one of the group of pathogenic bacterial species, pathogenic fungal species, pathogenic viral species, or pathogenic protozoal species. In a more preferred embodiment, the nucleic acid is complementary to the nucleic acid of a pretargeting composition. In certain preferred embodiments, the chelator moiety is an amidothiol chelator. Such kits are useful for the synthesis of radiolabeled compositions under mild conditions suitable for a variety of polymers. Further, radionuclides may be conjugated shortly prior to medical application, for formulation with pharmaceutically acceptable carriers (see *infra*).

In another aspect, the invention provides a kit comprising an antibody-chelator or a binding protein-chelator in a container, wherein the chelator comprises a sulfur atom protected by a protecting group of the formula -C(O)-lower alkyl (more preferably an acetyl group), and instructions for complexing the antibody-chelator or binding protein-chelator with a radionuclide under mild conditions. In preferred embodiments, the antibody-chelator or binding protein-chelator specifically binds to a tumor-specific sequence or a tumor-specific gene product. In preferred embodiments, the nucleic acid may carry the sequence complementary to tumor-specific DNA, which includes DNA from one of the class consisting of oncogenes, tumor promoters, tumor suppressors, cancer predisposition genes such as mutated Wilm's tumor or BrcA, or tumor marker genes.

In another embodiment, the invention provides libraries of chelator compounds, libraries of nucleic acid-chelators, and libraries of nucleic acid-chelator-radionuclides. Such libraries can be synthesized according to methods for combinatorial synthesis (see *infra*). Libraries of chelators, polymer-chelator (e.g., nucleic acid-chelator or protein-chelator) compounds, and polymer-chelator-radionuclides (e.g., nucleic acid-chelator-radionuclide or protein-chelator-radionuclide) are useful for rapidly screening for compounds with desired properties, e.g., low non-specific binding, selected lipophilicity, high or low affinity for radionuclides, and the like.

In a preferred embodiment, a library of chelator compounds comprises at least 10, more preferably at least 25, more preferably at least 50, more preferably at least 100, and still more preferably at least 500 different chelator compounds. In preferred embodiments, a library of polymer-chelator compounds comprises at least 10, more preferably at least 25, more preferably at least 50, more preferably at least 100, and still more preferably at least 500 different polymer-chelator compounds. In preferred embodiments, a library of polymer-chelator-radionuclides complexes comprises at least

10, more preferably at least 25, more preferably at least 50, more preferably at least 100, and still more preferably at least 500 different polymer-chelator-radionuclide complexes.

II. Methods of the Invention

In one aspect, the invention provides a method of synthesizing an activated ester of an S-protected mercaptoacetyl amino acid comprising the steps of (a) reacting an amino acid with an activated ester of an S-protected thioglycolic acid under conditions such that an S-protected mercaptoacetylamino acid and an activating alcohol are formed; and (b) reacting the S-protected mercaptoacetylamino acid and the activating alcohol with a coupling reagent under conditions such that an activated ester of an S-protected mercaptoacetyl amino acid is formed.

In preferred embodiments, the amino acid is an oligopeptide, more preferably a tripeptide, still more preferably triglycine. In certain preferred embodiments, the activated ester of S-acetylthioglycolic acid (SATA) is the N-hydroxysuccinimide (NHS) ester of S-acetylthioglycolic acid. In preferred embodiments, the activated ester of the S-protected mercaptoacetyl amino acid is an N-hydroxysuccinimide ester. In preferred embodiments, the coupling reagent is a carbodiimide, more prefereably dicyclohexylcarbodiimide. In preferred embodiments, the S-protected thioglycolic acid is SATA, e.g., the protecting group on sulfur is an acetyl group. In other preferred embodiments, the sulfur protecting group has the formula -C(O)-lower alkyl. In a preferred embodiment, step (b) includes the further step of providing additional activating alcohol to the reaction mixture formed in step (a) in a preferred embodiment, the activating alcohol is N-hydroxysuccinimide. It will also be appreciated that groups other than mercaptoacetyl groups, e.g., substituted mercaptoacetyl groups, can be used in a chelator moiety, e.g., by use of α-substituted thioglycolic acids to derivatize a tripeptide, thereby forming a substituted mercaptoacetyltripeptide.

The amino acid is preferably contacted with the activated ester of S-acetylthioglycolic in solution. In preferred embodiments, the solvent is a polar aprotic solvent, although any solvent capable of solubilizing the reactants without causing or participating in undesired side reactions can be used. Exemplary solvents include dimethylformamide (DMF), dichloromethane, dimethylacetamide, dioxane, tetrahydrofuran, ether, dimethoxyethane, and the like, or mixtures thereof. Reaction times will generally be in the range of 0.25 - 24 hours; progress of the reaction can be monitored by standard techniques, e.g., HPLC, thin-layer chromatography, NMR spectroscopy, and the like. The reactions can be performed at temperatures ranging from about 0°C to about 100°C, more preferably about 10°C to about 60°C, and more

preferably about 15°C to about 40°C. The reactions can also be performed under anhydrous conditions and inert atmosphere, e.g., of nitrogen or argon.

The method preferably includes the further step of purifying the S-protected mercaptoacetyl oligopeptide. Purification can be by a variety of means known in the art, including chromatographic methods such as flash chromatography, HPLC, or gel filtration chromatography.

The method provides advantages over known methods of synthesis of S-protected meraptoacetyl peptides. The synthesis requires only two steps, which can be performed in one pot, preferably without isolation or purification of intermediates. Thus, the inventive method is simple and rapid, and can provide high yields of the desired compounds. Furthermore, the starting materials (e.g., the NHS ester of SATA) are commercially available and are inexpensive. Activated esters of S-protected mercaptoacetyl peptides can be used as protected chelator moieties for synthesis of radionuclide-labeled molecules, e.g., polymers such as nucleic acids (including peptide nucleic acids), antibodies, polypeptides, carbohydrates, hormones, and the like. In many cases, covalent attachment of the chelator moiety to the target molecule (e.g., a polymer) can be easily and selectively achieved through methods known in the art for coupling of molecules with activated esters. In a preferred embodiment, the chelator moiety is covalently attached to the target via an amide linkage; hence, a molecule with a free amine group can be suitable as a target. In preferred embodiments the chelator moiety is attached to a polymer through a group such as an amide, an ester, or a thioester. In preferred embodiments, the chelator moiety is covalently bonded to a polymer through a moiety other than a thiourea. Other functionalities suitable for covalent attachment of a chelator moiety to a targeting molecule will be apparent to the skilled artisan.

In another aspect, the invention provides a method of synthesizing a polymer-chelator-radionuclide complex. The method includes the steps of contacting a polymer-chelator compound (e.g., polymers including nucleic acids (preferably PNA), proteins or polypeptides (e.g., antibodies) or carbohydrates) with a radionuclide under mild conditions, and allowing a polymer-chelator-radionuclide complex to form. In preferred embodiments, the polymer is a nucleic acid, e.g., a DNA, an RNA, a phosphorothioate or a PNA, more preferably a peptide nucleic acid. In preferred embodiments, the chelator moiety is a tetradentate chelator, more preferably an amidothiol, yet more preferably an oligopeptide-thiol, and still more preferably a tripeptide-thiol. A preferred embodiment of a chelator moiety is mercaptoacetyltriglycyl. In preferred embodiments, the efficiency of labeling of the chelator-polymer with radionuclide is at least 20%, at

least 30%, at least 40%, at least 50%, at least 60%, at least 70% or at least 80%. In preferred embodiments, the radionuclide is technetium-99m.

In preferred embodiments, the contacting step for radio-labeling occurs under conditions suitable for formation of a polymer-chelator-radionuclide complex. Thus, the radionuclide can be supplied in the form of a chelate, e.g., a tartrate or glucoheptonate complex, which can undergo transchelation with the macromolecule-chelator to form a macromolecule-chelator-radionuclide complex. Alternatively, the radionuclide can be supplied in a form which requires further treatment, e.g., reduction, to a chelatable form. In an exemplary embodiment, the method includes the step of contacting the radionuclide with a reducing agent to provide the radionuclide in a chelatable form. Thus, for example, pertechnetate can be reduced to Tc(V), e.g., $Tc(O)^{3+}$, which is then complexed by the chelator moiety. Preferred reducing agents include $SnCl_2$ and other reducing agents capable of reducing an oxidized radionuclide to a chelatable oxidation state, preferably under mild conditions.

It will be appreciated that the mild conditions of chelation, e.g., of temperature and pH, and the absence of harsh denaturants, can enable the user to complex a chelator-polymer to a radionuclide without significant denaturation and loss of structure and function of that polymer. In addition, postconjugation labeling can result in decreased radiation exposure of the personnel involved in clinical and research laborates and in shipping and handling, and the economy of decreased radionuclide procurement and waste. The invention provides a variety of custom-designed chelators that are suitable for various *in vivo* target organs, tissues, and compartments.

In another aspect, the invention provides methods for synthesizing libraries of chelator compounds. In yet another aspect, the invention provides methods for synthesizing libraries of polymer-chelator compounds. In still another aspect, the invention provides methods for synthesizing libraries of polymer-chelator-radionuclide complexes.

The synthesis of combinatorial libraries is well known in the art and has been reviewed (see, e.g., E.M. Gordon *et al.*, *J. Med. Chem.* 37:1385-1401 (1994)). The subject invention contemplates methods for synthesis of combinatorial libraries of chelator compounds, polymer-chelator compounds, and polymer-chelator-radionuclide complexes. Such libraries can be synthesized according to a variety of methods. For example, a "split-pool" strategy can be implemented in the following way for synthesis of a library of tripeptide mercapto acetyl chelators: beads of a functionalized polymeric support (e.g., a resin for peptide synthesis, e.g., Merrifield resin) are placed in a plurality of reaction vessels. To each aliquot of beads is added a solution of a different amino

acid (preferably protected as is conventional in peptide synthesis; see, e.g., M. Bodansky, "Principles of Peptide Synthesis," 2nd ed., Springer-Verlag (1991)), and the reactions proceed to yield a plurality of immobilized amino acids. The aliquots of derivatized beads are then washed, deprotected if appropriate, "pooled" (i.e., recombined), and the pool of beads is again divided, with each aliquot being placed in a separate reaction vessel. Two more cycles of amino acid addition re performed to yield a plurality of tripeptides, which can be derivatized to produce a library of mercaptoacetyl tripeptides. This library can then be screened, e.g., for lipophilicity, ease of removal of the protecting group, ability to chelate a radionuclide, and the like.

Other synthesis methods, including the "diversomer library" synthesis of Hobbs DeWitt *et al.* (*Proc. Natl. Acad. Sci. U.S.A.* 90:6909 (1993)), the "tea-bag" technique of Houghten (see, e.g., Houghten *et al.*, *Nature* 354:84-86 (1991)), and spatially-addressable arrays (see, e.g., Fodor *et al.*, *Science*, 251:767 (1991)) can also be used to synthesize libraries of compounds according to the subject invention.

Combinatorial libraries can be screened to determine whether any members of the library have a desired activity, and, if so, to identify the active species. Methods of screening combinatorial libraries have been described (see, e.g., Gordon *et al.*, *J Med. Chem.*, *op. cit.*). Soluble compound libraries can be screened by affinity chromatography, followed by identification of the isolated compounds by conventional techniques (e.g., mass spectrometry, NMR, and the like). Immobilized compounds can be also be screened by methods known in the art. Where libraries of immobilized compounds are screened to determine the ability of a chelator moiety to chelate a radionuclide, the immobilized compounds can be contacted with a radionuclide under complexing conditions, and the presence or absence of radioactivity can be measured to determine the chelating ability of the chelator moiety. A method for screening chelator compounds by conjugation with biocytin, followed by chelation with a radionuclide and immobilization on straptavidin, is described in the Examples, *infra*.

Combinatorial libraries of compounds can also be synthesized with "tags" to encode the identity of each member of the library (see, e.g., W.C. Still *et al.*, PCT Publication No. WO 94/08051). In general, this method features the use of inert, but readily detectable, tags, that are attached to the solid support or to the compounds. When an active compound is detected (e.g., by one of the techniques described above), the identity of the compound is determined by identification of the unique accompanying tag. This tagging method permits the synthesis of large libraries of compounds which can be identified at very low levels.

In another aspect, the invention provides a method of forming a polymer-chelator-radionuclide complex, comprising the steps of (a) contacting a polymer-chelator compound with a radionuclide under mild conditions; and (b) allowing a polymer-chelator-radionuclide complex to form. In preferred embodiments, the polymer-chelator compound is selected from the group consisting of protein-chelators and nucleic acid-chelators. In preferred embodiments, the radionuclide is technetium-99m. In preferred embodiments, the technetium-99m is provided in the form of a pertechnetate. In preferred embodiments, the method comprises further contacting the pertechnetate with a reducing agent. In a particularly preferred embodiment, reduction of the radionuclide and chelation of the reduced radionuclide occur in a single step, e.g., concomitantly. In preferred embodiments, the chelator is a mercaptooligopeptide chelator. In a preferred embodiment, the polymer-chelator is a peptide nucleic acid-chelator.

III. Pharmaceutical Compositions

The compounds of the invention can be incorporated into pharmaceutical compositions suitable for administration to a subject. In a preferred embodiment, the pharmaceutical composition comprises a polymer-chelator-radionuclide complex and a pharmaceutically acceptable carrier, and in another preferred embodiment, the pharmaceutical composition comprises a targeting moiety-chelator complex.

In yet another embodiment of the present invention, the pharmaceutical composition can be administered as part of a combination therapy, i.e., combined with other agents. For example, the combination therapy can include a composition of the present invention with at least one anti-cancer agent or at least one antibiotic, or incorporated into a protocol of several anti-cancer agents or antibiotics. Exemplary anti-cancer agents include cis-platin, adriamycin, and taxol. Exemplary antibiotics include isoniazid, rifamycin, and tetracycline. Further, the complexes of this invention can be delivered subsequent to a pretargeting composition.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Preferably, the carrier is suitable for intravenous, intramuscular, subcutaneous or parenteral administration (*e.g.*, by injection). Depending on the route of administration, the active compound may be coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound.

A "pharmaceutically acceptable salt" refers to a salt that retains the desired biological activity of the parent compound and does not impart any undesired

toxicological effects (see *e.g.*, Berge, S.M., *et al.* (1977) *J. Pharm. Sci.* 66:1-19). Examples of such salts include acid addition salts and base addition salts. Acid addition salts include those derived from nontoxic inorganic acids, such as hydrochloric, nitric, phosphoric, sulfuric, hydrobromic, hydroiodic, phosphorous and the like, as well as from nontoxic organic acids such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanoic acids, hydroxy alkanoic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts include those derived from alkaline earth metals, such as sodium, potassium, magnesium, calcium and the like, as well as from nontoxic organic amines, such as N,N'-dibenzylethylenediamine, N-methylglucamine, chloroprocaine, choline, diethanolamine, ethylenediamine, procaine and the like.

A composition of the present invention can be administered by a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. The active compounds can be prepared with carriers that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art. See, *e.g.*, *Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978. Such controlled release delivery formulations are preferred for particular pathologies, e.g. for delivery of an agent as a therapy for brain cancer.

The compounds can be orally administered, for example, with an inert diluent or an assimilable edible carrier. The compound (and other ingredients, if desired) may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the subject's diet. For oral therapeutic administration, the compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. The concentration of the compound in the compositions and preparations may, of course, be varied. The amount of compound in such therapeutically useful compositions is selected such that a suitable therapeutic dosage is obtained.

To administer a compound of the invention by other than parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. For example, the compound may

be administered to a subject in an appropriate carrier, for example, liposomes, or a diluent. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes (Strejan *et al.*, (1984) *J. Neuroimmunol.* 7:27).

Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Therapeutic compositions for delivery in a pharmaceutically acceptable carrier must be sterile, and preferably stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration.

The compositions of the subject invention comprising a novel chelator or polymer-chelator composition provide an alternative to prior art complexes which require "pre-conjugation" of radionuclides to chelators. The polymer-chelator molecules of the subject invention may be stored under standard conditions, and the radionuclide can be provided at a point prior to delivery into a subject, by transchelation under mild conditions. "Post-conjugation" chelation of radionuclide to polymer-chelator of the present invention yields greater economy and flexibility of radionuclide usage, and decreased exposure to radiation, over pre-conjugation chelation of radionuclide to chelator.

The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by microfilter sterilization.

Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Dosage regimens may be adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the disease situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals. The kits of the subject invention are suitable for formulation of the invented compositions in dosage unit form.

Examples of pharmaceutically-acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

For the therapeutic compositions, formulations of the present invention include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient which can be combined with a pharmaceutically acceptable carrier material to produce a single dosage form will vary depending upon the host being treated, and the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a

single dosage form will generally be that amount of the composition which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 0.01 per cent to about ninety-nine percent of active ingredient, preferably from about 0.1 per cent to about 70 per cent, most preferably from about 1 per cent to about 30 per cent.

Formulations of the invention suitable for oral administration may be in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of a compound of the present invention as an active ingredient. A composition of the present invention may also be administered as a bolus, electuary or paste.

Liquid dosage forms for oral administration of the compositions of the invention include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

Suspensions, in addition to the active compositions, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

Formulations of the present invention which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate, for example, to tumors or infections of the genito-urinary tract.

Dosage forms for the topical or transdermal administration of compositions of this invention include powders, sprays, ointments, pastes, creams, lotions, gels,

solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically-acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

The ointments, pastes, creams and gels may contain, in addition to a composition of this invention, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to a compound of this invention, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

Transdermal patches have the added advantage of providing controlled delivery of a compound of the present therapeutic inventions to the body. Such dosage forms can be made by dissolving or dispersing the composition in the proper medium. Absorption enhancers can also be used to increase the flux of the composition across the skin. The rate of such flux can be controlled by either providing a rate controlling membrane or dispersing the composition in a polymer matrix or gel. Devices, including patches, which transdermally deliver a composition by iontophoresis or other electrically-assisted methods can also be employed in the present invention, including, for example, the devices described in U.S. Patent Nos. 4,708,716 and 5,372,579.

Ophthalmic formulations, eye ointments, powders, solutions, drops, sprays and the like, are also contemplated as being within the scope of this invention, and are suitable for exigencies of pathological situations such as ophthalmic tumors or infections.

Pharmaceutical compositions of this invention suitable for parenteral administration comprise one or more composition of the invention in combination with one or more pharmaceutically-acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as

ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

The preparations of the present invention may be given orally, parenterally, topically, or rectally. They are of course given by forms suitable for each administration route. For example, they are administered in tablets or capsule form, by injection, inhalation, eye lotion, ointment, suppository, etc.; administration by injection, infusion or inhalation; topical by lotion or ointment; and rectal by suppositories. Injection (intravenous subcutaneous, or intraperitoneal) is preferred. For tumors or infections of the central nervous system, epidural administration of the therapeutic agents is preferred.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion.

These compounds may be administered to humans and other animals for therapy by any suitable route of administration, including orally, nasally, as by, for example, a spray, rectally, intravaginally, parenterally, intracisternally and topically, as by powders, ointments or drops, including buccally and sublingually.

Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically-acceptable dosage forms by conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of this invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.

The selected dosage level will depend upon a variety of factors including the activity of the particular compositions of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

In general, a suitable daily dose of a compositions of the invention will be that amount of the compound which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. It is preferred that administration be intravenous, intramuscular, intraperitoneal, or subcutaneous, administered perferably proximal to the site of the target. For example, for imaging of a cancer of the gastro-intestinal tract, oral administration to an unfed subject or patient is appropriate, while intravenous administration is appropriate for imaging of the urinary tract. Intraspinal administration is appropriate for imaging of the brain and central nervous system. The compounds of this invention for a patient, when used as an imaging agent, are preferred to be administered in the range of 0.1 milliCuries per kg of body weight to about 10 milliCuries per kilogram of body weight per day, more preferably from about 1 milliCurie per kg to about 4 milliCuries per kg.

If desired, the effective daily dose of a therapeutic compositions may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms.

While it may be possible for a compound of the present invention to be administered alone, it is preferable to administer the compound as a pharmaceutical formulation (composition).

Therapeutic compositions can be administered with medical devices known in the art. For example, in a preferred embodiment, a therapeutic composition of the invention can be administered with a needleless hypodermic injection device, such as the devices disclosed in U.S. Patent Nos. 5,399,163, 5,383,851, 5,312,335, 5,064,413, 4,941,880, 4,790,824, or 4,596,556. Examples of well-known implants and modules

useful in the present invention include: U.S. Patent No. 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; U.S. Patent No. 4.,486,194, which discloses a therapeutic device for administering medicants through the skin; U.S. Patent No. 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion rate; U.S. Patent No. 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; U.S. Patent No. 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments; and U.S. Patent No. 4,475,196, which discloses an osmotic drug delivery system. These patents are incorporated herein by reference. Many other such implants, delivery systems, and modules are well known to those skilled in the art.

In certain embodiments, the compounds of the invention can be formulated to ensure proper distribution *in vivo*. For example, the blood-brain barrier (BBB) excludes many highly hydrophilic compounds. To ensure that the therapeutic compounds of the invention cross the BBB (if desired), they can be formulated, for example, in liposomes. For methods of manufacturing liposomes, see, e.g., U.S. Patents 4,522,811; 5,374,548; and 5,399,331. The liposomes may comprise one or more moieties which are selectively transported into specific cells or organs ("targeting moieties"), thus providing targeted drug delivery (see, e.g., V.V. Ranade (1989) *J. Clin. Pharmacol.* 29:685). Targeting moieties may be used to pretarget (*supra*) a tumor or site of infection, prior to delivery of the radionuclide therapeutic complex. Exemplary targeting moieties include folate or biotin (see, e.g., U.S. Patent 5,416,016 to Low *et al.*); mannosides (Umezawa *et al.*, (1988) *Biochem. Biophys. Res. Commun.* 153:1038); antibodies (P.G. Bloeman *et al.* (1995) *FEBS Lett.* 357:140; M. Owais *et al.* (1995) *Antimicrob. Agents Chemother.* 39:180); surfactant protein A receptor (Briscoe *et al.* (1995) *Am. J. Physiol.* 1233:134); p120 (Schreier *et al.* (1994) *J. Biol. Chem.* 269:9090); see also K. Keinanen; M.L. Laukkanen (1994) *FEBS Lett.* 346:123; J.J. Killion; I.J. Fidler (1994) *Immunomethods* 4:273. In a preferred embodiment, the therapeutic compounds of the invention are formulated in liposomes; in a more preferred embodiment, the liposomes include a targeting moiety. In a most preferred embodiment, the therapeutic compounds in the liposomes are delivered by bolus injection to a site proximal to the tumor or infection.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases, the composition must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved

against the contaminating action of microorganisms such as bacteria and fungi. The vehicle can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, sodium chloride, or polyalcohols such as mannitol and sorbitol, in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate or gelatin.

Sterile injectable solutions can be prepared by incorporating the therapeutic compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the therapeutic compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient (i.e., the therapeutic compound) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

A "therapeutically effective dosage" preferably inhibits tumor growth or pathogen infection by at least about 20%, more preferably by at least about 40%, even more preferably by at least about 60%, and still more preferably by at least about 80% relative to untreated subjects. The ability of a compound to inhibit cancer or infectious disease can be evaluated in an animal model system that may be predictive of efficacy in human tumors and infectious diseases. Alternatively, this property of a composition can be evaluated by examining the ability of the compound to inhibit an activity *in vitro* by assays well-known to the skilled practitioner.

A therapeutically effective amount of a therapeutic compound can decrease tumor size, prevent or delay death of infected tissues or organs, decrease fever and white cell count, improve CD4 cell count, or otherwise ameliorate symptoms in a subject. One of ordinary skill in the art would be able to determine a therapeutically effective amount based on such factors as the subject's size measured by mass or surface area, the severity

of the subject's symptoms, and the particular composition or route of administration selected.

In the compositions of the present invention, the nucleic acid moieties of the subject compositions are preferably chemically synthesized. However DNA and RNA are also available from biological sources, for example, produced in reasonably high yields from a recombinant organism in which the gene for the DNA is cloned, or in which the RNA is transcribed by a high activity promoter of a cloned gene. In this manner either the sense strand or the antisense strand is obtained, according to the objectives of the skilled artisan practicing this invention. Whether the source of the nucleic acid is from chemical synthesis or a biological sample, the material is preferably single stranded, and purification methods known to the skilled artisan, for example acrylamide gel electrophoresis or column chromatography for example on a P4 column (BioRad, Melville, NY), are used to obtain isolated nucleic acids for covalent coupling to the chelator of the invention. Covalent linkage of the chelator molecule can be achieved by coupling, e.g., via an amine attached to the terminal phosphate group through a 6-member methylene carbon spacer. Attachment of a biotin compound moiety, if appropriate (*J. Nuclear Med.* 36: 2306-2314), can be achieved, e.g., directly or through a 15-member amide-polyether to the terminal phosphate. Other entities that would enhance the therapeutic function of the nucleic acid are similarly linked to a terminus of the nucleic acid, for example a member of the cytokine class of proteins such as epidermal growth factor, platelet-derived growth factor, and nerve growth factor.

The invention comprises a variety of oligopeptide tetradentate chelators, preferably tripeptide, composed of, for example, but not limited to, combinations of the amino acids gly, ser, ala, leu, ile, phe, val, pro, met, phe and thr, which offer the user of the invention an array of chelators which embody a variety of desired characteristics, and is not limited to any one amino acid moiety. The MAG_3-radionuclide complex, for example, is stable in serum, as it is exemplified in the Example below, so that a smaller proportion of the radionuclide is transferred non-specifically to serum proteins than with other chelators such as SHNH (see Example 7). Greater *in vivo* stability produces greater contrast as an imaging agent between the target tumor or site of infection, or organ to be imaged such as the kidney, and the background of non-target tissues. A greater therapeutic index is obtained, i.e., fewer side effects due to destruction of normal tissue, are observed if nonspecific transfer or loss of the radionuclide metal occurs. Further, choice of an alternative amino acid to glycine, such as phenylalanine with a large hydrophobic side chain, for the oligopeptide chelator synthesis to produce MAG_2F, enables the user to custom design properties to improve a desired activity as

cell permeation, so that the imaging and therapeutic aspects of the subject composition of matter are further enhanced.

The invention provides a method for imaging a tumor, comprising the steps of administering to a patient a radionuclide-chelator-peptide nucleic acid composition in a pharmaceutically acceptable carrier, and detecting a radioactive signal to detect a tumor. In a preferred practice of the invention, the nucleic acid sequence of the composition used for this method is complementary to a tumor-specific sequence that is uniquely expressed in the tumor. As described herein, such a sequence may be in an oncogene, a mutated tumor suppressor gene, a form of a gene that confers predisposition to a cancer, or a gene for a protein marker that appears on a tumor. In a more preferred method, the base sequence of the nucleic acid is complementary to that of a nucleic acid of a pretargeted composition. Examples of these are well-known to those skilled in the art, and are given here for the purposes of illustration, without limiting the present invention to these examples.

The nestin protein, which is expressed in normal mammalian fetal development, is expressed on tumors of the central nervous system, including most forms of brain cancer (McKay, D.G. Ronald, *U.S. Patent No. 5,338,839*, 8/16/94). It is also expressed on melanomas and on metastasized melanomas (V.A. Florenes, R. Holm, O. Myklebost, U. Lendahl, O. Fodstad, *Cancer Res. 54*: 354-6, 1994), which can metastasize to other tissues and are thus difficult to detect and cure. The methods of the invention include administration of, for example, PNA of a sequence complementary to the mRNA of the nestin protein, preferably to the unique portion of the human nestin gene, to target the PNA-chelator-radionuclide composition to the brain tumor, or to the metastasized melanoma cells, and localize the radioactivity to these sites. In a more preferred method, the base sequence of the nucleic acid is complementary to that of a nucleic acid of a pretargeted composition. The preferred site of delivery is within the central nervous system or directly to the brain via spinal injection or fine needle delivery, and the tumors may be imaged with equipment standard at a clinical nuclear medicine facility, for example, a whole body scanner (Siemans). Preferably, the whole body scanner is equipped with computing equipment and software, such as a Harris Computer Center with multiple terminals permitting programming and the use of data reduction programs. The radioactivity accumulating a specific sites in the body can be converted to images of the tumor with the associated software. Methods and routes for detection of nestin DNA sequence information for brain cancer can be applied to diagnostics and imaging of meningiomas, another pathological condition associated with the nervous system, using data available on the identification and characterization of genes differentially expressed

in mengiomas (M. Murphy, M.J. Pykett, P. Harnish, K.D. Zang, D.L. George, *Cell Growth Differ 4*:715, 1991).

Other tumor types for which the methods of this invention are applicable include, but are not limited to, Wilm's tumor (A.J. Buckler, K.M. Call, T.M. Glaser, D.A. Haber, D.E. Housman, C.Y. Ito, J. Pelletier, Rose, E.A. Rose, U.S. Patent No. 5,350,840,), a pediatric kidney cancer due to a somatic mutation in the patient's single copy of a gene normally found in two intact copies. Wilm's tumor can be cured surgically in 95% of cases, but an imaging agent is appropriate for monitoring remission, and for detection and imaging of newly appearing tumors. Other examples of known cancer-associated DNA sequences for which the compositions of matter and methods of the current invention are suitable include those associated with gastrointestinal cancer (R. Fishel, R.D. Kolodner, R.A.G. Reenan, R.A. Reenan, World Patent No.WO 95/14085, 05/26/95), those associated with appearance of multiple drug resistance during chemotherapy (J.M. Croop, P. Gros, D.E. Housman, U.S. Patent No.5,198,344), and to those associated with a large number of oncogenes such as Rb, ras, c-myc and neu, the sequences of which are readily available to those with skill in the art.

The inventive compositions and methods for diagnosis, imaging and therapeutic applications are tools to be used in combination with standard medical compositions and methods, such as surgery, chemotherapy, and radiation therapy for treating cancer. Further, the inventions of this application, while described as diagnostic or therapeutic, may in fact serve both purposes, for example a therapeutic dose of radionuclide also serves as a tool to monitor the course of therapy. For example, accumulation of radiolabel at a site of a tumor or an infection can be monitored for maintenance of size and shape over the course of the therapy without necessity of a separate dosage merely for diagnostic imaging. The known half-life of the radionuclide permits calculation of a schedule by which the labeling density will decline from its initial value. A greater decrease in size of the tumor or infected site indicates effectiveness of the treatment, i.e., a decline in label density at a rate faster than that of the half-life of the radionuclide.

Similarly, the compositions and methods of the current invention are useful for diagnostics, imaging and for therapeutic agents for infectious diseases of humans, animals and plants. The term "infectious disease" is meant to include disorders caused by one or more species of bacteria, viruses, fungi, and protozoans, which are disease-producing organisms are collecively referred to as "pathogens." In this invention, pathogens are exemplified, but not limited to, *Mycobacterium tuberculosis*, *M. leprae*, *Pseudomonas aeruginosa*, *Shigella dysenteria*, *Salmonella typhi*, *S. paratyphi*, *Streptococcus hemolyticus*, *Hemophilus pneumoniae*, *Escherichia coli* serotype 0157,

Chlamydia species, *Helicobacter* species; HIV-1,-2, and -3, HSV-I and -II, non-A non-B, non-C hepatitis virus, pox viruses, rabies viruses, *Aspergillus* species, *Entamoeba histolytica*, *Giardia* species, *Erwinia carotovora*, cauliflower mosaic virus, and Newcastle disease virus. Obtaining unique sequences from these organisms by screening available data bases and by performing hybridizations *in vitro* are commonly known to those skilled in the art (see, e.g., Ladner et al, U.S. Patent No. 5,096,815, March 17,1992). Further, targets such as antigens associated with each pathogen are known, and specific antibodies, antibody fragments, and binding proteins are used to design targeting and pretargeting agents, complexes, molecules, and compositions.

In compositions of this invention, the nucleotide sequences of interest can be complementary to tumor-specific mRNA (or to a unique sequence from a pathogen), so that the nucleic acid of the composition is doubly functional as a "homing" or delivery molecule for the target tumor or infected cells, but also may possess a therapeutic function in that it forms a stable complex with the naturally occuring mRNA, or with genomic DNA to inhibit transcription.

The specific activity of a radionuclide pertains to the ratio of molecules of composition bearing technetium-99m or equivalent (*supra*) to the total number of molecules of the composition. The high coupling efficiencies described in the examples below indicate that the specific activity of the radionuclide is sufficiently high for use as a therapeutic, diagnostic, or imaging composition.

Exemplification

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

The chelator N-[N-[N-[(benzoylthio)acetyl]glycyl]glycyl]glycine (benzoyl MAG_3) has been used successfully to radiolabel proteins and other molecules with technetium-99m and radiorhenium. Prior to radiolabeling, the sulfur in this mercaptotripeptide chelator has previously been protected by a benzoyl leaving group which requires extreme alkaline pH or boiling temperatures for deprotection. As a result, the benzoyl-protected chelator is generally deprotected and radiolabeled prior to conjugation (i.e. preconjugation labeling) in the case of carriers such as proteins or polypeptides which cannot withstand the harsh deprotection conditions. The examples below demonstrate a one-pot, two-step, synthesis of the N-hydroxysuccinimide derivative of acetyl-MAG_3 (NHS-MAG_3) with commercially available reagents. Good

labeling efficiencies of macromolecules with technetium-99m can be achieved rapidly at near-neutral pH and at room temperature with NHS-MAG_3. Technetium-99m labeled MAG_3-DNA was evaluated for stability in comparison to the same DNA radiolabeled with technetium-99m via a known chelator, hydrazino nicotinamide (SHNH). The radiolabel in these complexes was found to be similarly stable to transchelation to cysteine. However, in contrast to SHNH-DNA, little serum protein binding of the labeled MAG_3-DNA was observed. Since binding to proteins in serum and in tissue as a major drawback in the use of oligonucleotides labeled with technetium-99m via the chelator SHNH, the MAG_3 chelator provides certain advantages for radiolabeling these biomolecules.

Example 1 describes the synthesis of acetyl-MAG_3 by reacting triglycine with SATA for 15 min at room temperature. The acivated ester NHS-acetyl-MAG_3 is then prepared, without a purification step, from the first reaction product by DCC-mediated coupling to MAG_3 of the *in situ*-generated N-hydroxysuccinimide, as shown in Example 2. Example 3 describes coupling of NHS-acetyl-MAG_3, purified only by centrifugation, to a 22-base single-stranded DNA through an amine on the 3' end, by reaction for 15 min at room temperature. Example 4 describes radiolabeling by transchelation from technetium-99m-tartrate, and in this example, a yield of 84 + 6% from 5 different experiments and specific activity of 70 microCuries per microgram is obtained within 15 min at room temperature. Example 5 establishes that the radionuclide is quantitively associated with DNA, that DNA thus labeled can bind its complementary nucleic acid strand, and that an attached biotin moiety is capable of binding avidin. Example 6 uses cysteine challenge to show that the radionuclide-chelator complexes with each of SHNH-DNA and MAG_3-DNA conjugates are stable. Example 7 shows that the MAG_3 chelate of technetium-99m on DNA has less nonspecific binding to serum protein than the SHNH chelate.

Example 8 describes the labeling of PNA which is more stable than the relatively labile DNA. Example 9 shows that hybridization of a PNA-MAG_3-radionuclide complex to its complementary sequence occurs rapidly, and is complete within 10 min. In Example 10, the PNA-MAG_3-radionuclide composition is stable in serum, and little is associated with serum protein or is catabolized or associated with cellular material. This composition, when injected into mice, is stable in the urine as shown by Example 11. The organ distribution found in mice is discussed in data described in Example 12. Example 13 presents use of PNA as a pretargeting moiety and for radionuclide targeting in a mouse model of infection, and a mouse tumor model is given in Example 14. Example 15 shows synthesis of additional novel chelators.

In the following examples, solutions were sterilized by terminal filtration through a 0.22 micrometer filter (Gelman, Ann Arbor, MI), and sterile pipette tips (Brinkmann, Westbury, MA) were used. All tubes, etc. were autoclaved prior to use.

Example 1. Synthesis of acetyl-MAG_3 As shown schematically in Fig. 1, acetyl-MAG_3 was synthesized by reacting triglycine with S-acetylthioglycolic acid N-hydroxysuccinimide ester (SATA) for 15 min at room temperature. S-acetylthioglycolic acid N-hydroxysuccinimide ester (SATA) was obtained from Sigma Chemical Co., St. Louis, MO and was used without further purification. To 0.97 milliliters of a 0.225 M NaOH was added 50 milligrams of triglycine (264 micromol) and 10 microliters of a freshly-prepared 50 mM EDTA. This solution was passed through a 0.2 micrometer filter to remove amine-containing particulates. A solution of 90 milligrams (390 micromol) of SATA in 340 microl of DMF (dried over molecular sieves) was prepared and was added dropwise to the stirred triglycine solution. After 15 min of stirring at room temperature, the non-aqueous solution was adjusted from an apparent pH of 8.9. to an apparent pH of approximately 2.7 (measured with a glass electrode-pH meter) by the addition of 37.6 microliters of 6 M HCl. An initial pH of about 8.9 was selected to deprotonate the amine on triglycine (pK 7.9) but without reaching extreme basic pH values in which the acetyl group on SATA may hydrolyze. The pH was lowered as soon as possible to minimize hydrolysis of the acetyl group. NMR and melting point determinations for the products of this reaction are described below with the products for the second reaction, in Example 2.

Example 2. Synthesis of NHS acetyl-MAG_3. Without purification, NHS-MAG_3 is prepared directly from the reaction of Example 1, usually in 15 hrs but in as little as 1-2 hrs by dicyclohexylcarbodiimide (DCC)-mediated coupling to acetyl-MAG_3 of the in situ-generated N-hydroxysuccinimide. Dicyclohexylcarbodiimide (DCC) is obtained from from Sigma Chemical Co. (St. Louis, MO) and used without further purification. A solution of 60 milligrams (290 micromol) of DCC in 3.6 milliliters of dry DMF is added rapidly to the stirred triglycine/SATA solution (apparent pH of about 5.0). The solution becomes cloudy within 2 min as dicyclohexylurea precipitates. The reaction is stirred at room temperature in the dark for 2-4 hrs and is then cooled to -20°C for an additional hour for maximum precipitation. After centrifugation at 4°C, 2500 g for 15 min, the clear supernatant is removed.

Due to the presence of water in the DMF solution, the NHS-acetyl-MAG_3 preparation in this form is used within 24 hrs of preparation. For long-term storage, the

NHS-acetyl-MAG_3 water/DMF solution is evaporated to near-dryness in 15-30 min on a rotary flash evaporator (Rotavapur-R, Buchi, Switzerland) and then lyophilized to dryness within 1 hr on a lyophilizer (Virtis, Garden NY). After drying in this fashion, the NHS-acetyl-MAG_3 can be stored indefinitely at room temperatures in a dessicator When using the dry, powdered NHS-acetyl-MAG_3 for conjugation, an arbitrary value of 50% by weight was assumed for its purity.

The results of the reactions in Examples 1 and 2 were assessed by NMR analysis and melting point determinations. For these analyses, the MAG_3 carboxylate intermediate and the NHS-acetyl-MAG_3 were each purified on an open column silica gel column (Silica Gel 60, 0.2-0.5 mm particle size, EM Science, Gibbstown NJ) using acetonitrile as eluant. The NMR analyses were performed in fully deuterated DMSO on a 300 MHz instrument (Varian Unity-300, Varian Associates Inc., San Fernando, CA). The chemical shifts for S-acetyl MAG_3 were 2.36 (s,3H), $SCOCH_3$; 3.66 (s,2H), $COCH_2S$; 3.72-3.78 (m, 6H), NCH_2CO); 8.15-8.36 (m,3H), NHCO. The chemical shifts for NHS-acetyl-MAG_3 were 2.38 (s,3H), $SCOCH_3$; 2.80 (s,4H), succinimidyl; 3.68(s,2H), $COCH_2S$; 3.70-3.80 (m, 6H), NCH_2CO); 8.20-8.38 (m,3H), NHCO.

The uncorrected melting points were determined (Mel-Temp, Laboratory Devices, Cambridge, MA) to be 210-212 °C (dec.) for acetyl-protected MAG_3 and 148-151 °C for NHS-acetyl-MAG_3.

Example 3. DNA Conjugation with MAG_3 and SHNH. Two 22-base single-stranded DNA oligonucleotides were purchased from a commercial supplier (Operon Technologies, Alameda, CA). Aliquots of 20-1000 µg of DNA were added to sterile plastic vials which were immediately frozen at -20°C for storage. Reagent-grade avidin (Molecular Probes, Eugene, OR), disodium ethylenetriaminetetraacetic acid (EDTA) (Aldrich Chemical Co., Milwaukee, WIS), D-biotin, dicyclohexylcarbodiimide (DCC), L-cysteine, dimethylformamide (DMF), sodium glucoheptonate, sodium tartrate, tricine, triglycine, streptavidin was obtained from Sigma Chemical Co., St. Louis, MO, and was used without further purification. The base sequences were 5'-biotin-TA ATA CGA CTC ACT ATA GGG AG-amine-3' and its complement, as described previously (Hnatowich D.J., Winnard P. Jr., Virzi F, Fogarasi M, Sano T, Smith CL, Cantor CR, Rusckowski M, *J. Nucl. Med.* 36: 2306-2314, 1995). A biotin moiety was attached via a 15-member amide-polyether linker to the terminal phosphate on the 5' end while a primary amine was attached to the terminal 3' phosphate group via a 6-member methylene carbon spacer. The molecular mass was about 8 kDa. The melting temperature in physiological saline was calculated to be 62°C for double-stranded DNA

(Wetmur, J.G., Rev Biochem Mol Biology 26: 227-259; 1991). The DNAs were purchased unpurified and were used without further purification. After coupling, the conjugate could be stored for extended periods since the attached MAG_3 group was still protected.

For conjugation with MAG_3, a solution of single-stranded amine-derivatized DNA (100 - 1000 microgram) was prepared at a concentration of 2 milligrams/milliliters in 0.25 M $NaHCO_3$-1 M NaCl-1 mM EDTA, pH 8.5. The DNA solution was heated to 60-70 °C for 5-10 min to dissociate any DNA duplexes and immediately plunged into ice water. The DMF/water solution of NHS-acetyl-MAG_3 was then added to the stirred DNA solution to a MAG_3 : DNA molar ratio estimated to be 20 : 1. This solution was incubated at room temperature for 15 min in the dark. The conjugation of the same oligonucleotides with SHNH was achieved by reacting the NHS derivative of SHNH with the DNA primary amine as has been previously described (Hnatowich D.J., Winnard P. Jr., Virzi F, Fogarasi M, Sano T, Smith CL, Cantor CR, Rusckowski M., J Nucl Med 36:2306-2314, 1995) and was similar to that described above for MAG_3 conjugation. The DMF solution of NHS-SHNH was added, while vortexing, to the DNA solution until a final molar ratio of SHNH : DNA of 25 : 1 was reached. The solution was incubated at room temperature in the dark for 1 hr.

For both methods, the conjugated DNAs were purified on a 0.7 x 20 cm P4 column (BioRad, Melville, NY) eluted with 0.25 M NH_4Acetate- 0.25 mM DTPA, pH 5.2 buffer. The DTPA was added to help prevent radiocolloid formation during radiolabeling. Fractions (0.4 milliliters) off the P4 column were collected and the absorbency (260 nm) of each measured (Hitachi Instruments, Danbury, CT). Oligonucleotide concentrations were estimated using an extinction coefficient at 260 nm determined in this laboratory of 30 microliters/micrograms for a 0.1% solution. The absorbency of SHNH and MAG_3 under these conditions was found to be negligible. After purification, the concentration of DNA in the peak fractions was typically about 1 milligrams/milliliters for a conjugation of 1000 microgram of DNA. Both modified DNAs were stored at -20°C for at least three months.

Example 4. Oligonucleotide Labeling with Technetium 99m. Preliminary labeling studies comparing tricine, glucoheptonate and tartrate as transchelator were performed at 22-100°C and at pH values between 5.5-7.6. Since superior labeling efficiencies were consistently achieved with tartrate at room temperature, pH 7.6, the MAG_3-conjugated oligonucleotides were eventually radiolabeled with technetium-99m by transchelation exclusively from labeled tartrate (Fritzberg A.R., Nuklearmedzin 1987; 26: 7-12). The

technetium-99m-pertechnetate was obtained from a ^{99}Mo-technetium-99m radionuclide generator (Dupont, Billerica, MA). Succinimidyl 6-hydrazinonicotinate-SHNH was obtained from Dr. M. Abrams (Johnson Matthey Inc., West Chester, PA).

A fresh 50 milligrams/milliliters solution of sodium tartrate was prepared in sterile 0.5 M $NaHCO_3$, 0.25 M NH_4Acetate, 0.18 M NH_4OH, pH 9.2. The high pH of the tartrate solution was necessary so that the final pH would be approximately 7.6. In addition, a 1.0 milligrams/milliliters solution of $SnCl_2.2H_20$ in 10 milliM HCl was prepared just prior to use.

To a sterile test tube containing the acetyl-MAG_3-DNA (about 10-100 micrograms, 10-100 microliters) was added sufficient technetium-99m-pertechnetate solution (2-10 microliters) to provide about 100 uCi/ug of DNA. To this was added the tartrate solution to a final concentration of about 6-7 ug/microliters. The stannous ion solution was added immediately thereafter and such that 1 microgram of $SnCl_2.2H_2O$ was added for each 10 µg of DNA. Higher activities of technetium-99m required proportionately larger volumes of the tin solution.

After 15 min at room temperature, the labeled DNA was purified on a 0.7 x 20 cm gel filtration column of Sephadex G-25 using sterile 0.25 M NH_4Acetate, pH 5.2, or saline, as eluant. Radioactivity and absorbency at 260 nm were used to identify and quantitate peak fractions. Preparations were routinely analyzed by size exclusion HPLC using a single 1 x 30 cm Superdex 200 column (Pharmacia, Piscataway, NJ). The recovery was routinely recorded. Control labeling was performed in which the native, unconjugated DNA was subjected to the identical labeling procedure to assess the extent of nonspecific labeling.

The SHNH-conjugated oligonucleotides were radiolabeled with technetium-99m by transchelation from tricine as previously described (Hnatowich D.J., Winnard P. Jr., Virzi F, Fogarasi M, Sano T, Smith CL, Cantor CR, Rusckowski M., J Nucl Med, 36: 2306-2314, 1995; Hnatowich D.J., Mardirossian G., Fogarasi M. Sano T, Smith CL, Cantor CR, Rusckowski M, Winnard P. Jr., J. Pharmacol. Exp Ther 276:326-334, 1996). Briefly, a solution of tricine was prepared at neutral pH to which was added a fresh solution of $SnCl_2$. The technetium-99m-pertechnetate solution was then added to the DNA-tin solution and, immediately, a 20-microliters aliquot of the tin-tricine solution was added. After 10 min at room temperature, the labeled DNA was purified on a 0.7 x 20 cm gel filtration column of Sephadex G-50 using sterile 0.15 M saline as eluant. The radiochemical purity was determined by HPLC as described above for the labeling of MAG_3-DNA.

The technetium-99m labeled MAG_3-DNA was evaluated for stability in comparison to the same DNA radiolabeled with technetium-99m via the hydrazino nicotinamide (SHNH) chelator . The radiolabel is similarly stable to transchelation to cysteine. However, in contrast to SHNH-DNA, no evidence for serum protein binding of the labeled MAG_3-DNA is observed.

Optimum labeling conditions were consistently obtained with tartrate as transchelator. Under the set of conditions described above, labeling efficiencies of 84% (s.d. 6%, five separate experiments) were achieved within 15 min at room temperature. Neither raising the temperature or increasing the labeling time greatly improved the labeling efficiency. Specific activities of up to 70 microCuries/microgram were obtained. Control labeling of unmodified DNA under these conditions showed about 0-2% binding.

DNAs which had been coupled with MAG_3 have been radiolabeled without loss of efficiency after more than six months of storage at -20°C. These data illustrate that high labeling efficincies of MAG_3-PNA can be achieved using mild conditions. That the conjugated DNA could be successfully radiolabeled after storage at refrigerator temperatures for more than 6 months strongly suggests that the sulfur was still protected and that the deprotecting group is hydolyzed away during labeling, possibly by the excess stannous ion present to reduce the technetium-99m-pertechnetate.

Example 5. HPLC Studies. In addition to measuring radiochemical purity, size exclusion HPLC analysis was also used to establish whether the conjugation and labeling procedure had diminished the ability of the labeled DNA to bind to avidin through the biotin moiety of the DNA and to hybridize with its complementary single-stranded DNA. The radiolabeled MAG_3-DNA was analyzed by HPLC before and after the addition of avidin and before and after the addition of the complementary DNA bound to avidin (in which all unoccupied biotin sites were saturated with D-biotin).

To test the ability of the labeled DNA to bind to avidin through its biotin moiety, a four-fold molar excess of avidin was added to a solution of labeled DNA. After 1 hr, the solution was reanalyzed by HPLC. As a control, the identical analysis was also performed after the addition of biotin-saturated avidin to the labeled DNA.

To test the ability of the labeled DNA to hybridize to its complement, the complementary unlabeled DNA-avidin construct was first prepared by adding the complementary DNA at a four-fold molar excess to avidin and allowing 1 hr at room temperature for the binding of DNA through its biotin moiety . The remaining biotin-binding sites were then blocked by the addition of a 10-fold molar excess of D-biotin.

The preparation was purified over a 0.7 x 20 cm column of Sephadex G75 using sterile 0.25 M NH_4Acetate-0.5 M NaCl pH 7.2 buffer as eluant.

Hybridization was accomplished by incubating 0.4 microgram of the labeled DNA in saline with a 4-fold molar excess of the complementary DNA-avidin preparation. After 1 hr at room temperature, the unpurified solution was analyzed by HPLC. The identical study was performed with the biotin-saturated complementary DNA-avidin preparation.

Size exclusion HPLC radiochromatograms were obtained on a single 1 x 30 cm Superdex 200 column (Pharmacia, Piscataway, NJ) and 0.1 M sodium phosphate, pH 7 eluant.

Figure 2 presents several radiochromatograms obtained by size exclusion HPLC analysis. Panel A is that of the radiolabeled DNA itself. Panel B is the result of adding the labeled DNA to biotin saturated avidin. In this case, the absence of a shift to higher molecular weight suggests the absence of nonspecific binding of the labeled DNA to avidin. Panel C is the result of adding the labeled DNA to unsaturated avidin. In this case, the pronounced shift in radioactivity to higher molecular weight is the result of binding of the DNA to avidin through its biotin moieties. The shift indicates that the radiolabel is on the DNA as expected and that the conjugation and labeling procedures did not affect the biotin moiety in its affinity for avidin. The partial shift is most likely explained by assuming that approximately half the DNA molecules were obtained without the biotin group attached.

Lastly, Panel D shows the result of adding the labeled DNA to avidin to which the complementary DNA had previously been bound through its biotin moiety. In this case, the shift is quantitative, confirming that the radiolabel is on the DNA and that the conjugation and labeling procedures did not affect the ability of the DNA to hybridize under the conditions of this measurement.

Example 6. Cysteine challenge. The stability of technetium-99m-MAG_3-DNA to cysteine transchelation compared to that of technetium-99m-SHNH-DNA was evaluated at one cysteine concentration and after 1 hr in 37°C 25 milliM NH_4Acetate, pH 7.0 buffer. Both labeled DNAs were added at a final concentration of 0.4 mM to a solution of l-cysteine in 25 mM NH_4Acetate, pH 7.0 such that the cysteine : DNA molar ratio was 650 : 1 (Hnatowich DJ, Virzi F, Fogarasi M, Winnard P. Jr., Rusckowski M, Nucl Med. Biol 1994; 21: 1035-1044). After an incubation period of 1 hr in a 37 °C water bath, samples were removed for size exclusion HPLC analysis. The area under the DNA

and cysteine peaks in the radiochromatographic profiles were evaluated and compared between the two labeling methods.

Figure 3 presents radiochromatograms obtained by size exclusion HPLC analysis of the same single-stranded phosphodiester DNA labeled with technetium-99m via both SHNH and MAG_3. In the case of both labeling methods, panel A presents the radiochromatograms of the radiolabeled DNAs themselves. Panel B is the result of incubating the radiolabeled DNAs at 37°C with cysteine at a 650 molar excess for 1 hr. Under the conditions of this analysis, radiolabeled cysteine appears as a peak in fraction 75 (Hnatowich DJ, Virzi F, Fogarasi M, Winnard P. Jr., Rusckowski M., Nucl Med. Biol 1994; 21: 1035-1044). A small peak may be present in the radiochromatogram of MAG_3; nevertheless the stability of both the SHNH and MAG_3 chelates towards transchelation to cysteine is apparent. This indicates that the radionuclide-chelator complexes with each of the chelators are stable.

Example 7. Serum Incubation Studies. Labeled MAG_3-DNA was incubated at a concentration of 10 micrograms/milliliters in fresh human serum at 37 °C. Samples were periodically removed over 24 hrs for size exclusion HPLC analysis.

The last panel (C) in Fig. 3 shows the result of analyzing serum samples into which the labeled DNA were added. In the case of the SHNH chelator after 1 hr of incubation at 37°C, the technetium-99m radiolabel is primarily on serum proteins as shown by the shift to higher molecular weight (earlier fractions). This property has been observed previously for DNAs radiolabeled with technetium-99m via the SHNH chelator (Hnatowich D.J., Winnard P. Jr., Virzi F, Fogarasi M, Sano T, Smith CL, Cantor CR, Rusckowski M., J Nucl Med 36: 2306-2314, 1995; Hnatowich D.J., Mardirossian G., Fogarasi M. Sano T, Smith CL, Cantor CR, Rusckowski M, Winnard P. Jr., J. Pharmacol. Exp Ther 276: 326-334, 1996). In the MAG_3 case, by contrast, very little serum protein binding is evident. In its stead, several lower molecular weight peaks are present and are likely due to nuclease digestion of the single-stranded phosphodiester DNA. Similar low molecular weight catabolites have been observed previously in the case of DNA radiolabeled with ^{111}In under conditions which also minimized serum protein binding (Hnatowich D.J., Winnard P. Jr., Virzi F, Fogarasi M, Sano T, Smith CL, Cantor CR, Rusckowski M, J Nucl Med 36: 2306-2314 1995).

The properties of technetium-99m labeled MAG_3-DNA in 37°C serum over 24 hrs are shown in Fig. 4. After 24 hrs of incubation, very little intact labeled DNA remains with the activity present primarily the lowest molecular weight catabolite peak as seen in previous results.

Experience with the SHNH chelator for antibody labeling (Abrams M.J., Juweid M., tenKate C.I., Schwartz D.A., Hauser M.M., Gaul F.E., Fuccello A.J., Rubin R.H., Strauss H.W., Fischman A.J., J Nucl Med 31: 2022-2028, 1990.; Hnatowich D.J., Mardirossian G., Ruscowski M., Fogarasi M, Virzi F, Winnard P Jr., J Nucl Med 34; 109-119, 1993) has shown that technetium-99m can be attached effectively and stably in this manner. However, high molecular weight aggregates have been observed and have been attributed to peculiarities of the technetium-99m-SHNH chelate (Hnatowich D.J., Mardirossian G., Ruscowski M., Fogarasi M, Virzi F, Winnard P Jr., J Nucl Med 34; 109-119, 1993). Whereas this property may not interfere seriously in the case of high molecular weight species such as protein, it may become more serious when peptides and smaller molecules are labeled. That the same single-stranded DNA displays minimal tendency to bind nonspecifically to serum proteins when labeled with ^{111}In via a DTPA chelate, but is strongly bound to protein when labeled with technetium-99m via SHNH (Hnatowich D.J., Winnard P. Jr., Virzi F, Fogarasi M, Sano T, Smith CL, Cantor CR, Rusckowski M, J Nucl Med 36: 2306-2314, 1995). This property influences the biodistribution of technetium-99m when administered to mice on a SHNH-derivatized DNA, (Hnatowich D.J., Mardirossian G., Fogarasi M. Sano T, Smith CL, Cantor CR, Rusckowski M, Winnard P. Jr., J. Pharmacol. Exp Ther 276: 326-334 1996).

For consideration of alternative chelators to SHNH for the labeling of oligonucleotides with technetium-99m, the S-acetyl MAG_3 tripeptide chelator is compared in these Examples. This chelator binds technetium-99m stably as the free chelate for kidney function investigations (Fritzberg AR., Kasina S., Eshima D., Johnson D.L, J Nucl Med 27: 111-116; 1986) and when conjugated to proteins such as antibodies, (Fritzberg A.R., Berninger R.W., Hadley S.W. *et al.*, Pharmaceutical Res. 5: 325-334; 1988. No evidence of nonspecific protein binding through this chelate has been reported.

One prior use of this chelator involved protection of the sulfur atom with a benzoyl group, which requires temperatures of up to100°C for hydrolysis (Fritzberg A.R., Berninger R.W., Hadley S.W. *et al.*, Pharmaceutical Res. 5: 325-334; 1988, Bioconjugate Chem 1: 431-437; 1990; Goldrosen MH., Biddle WC., Pancook S. Bakshi S., Vanderheyden J-L., Fritzberg A.R., Morgan A.C., Foon K.A., Cancer Res. 50: 7973-7978; 1990) and is therefore less suitable for use in radiolabelling temperature-sensitive molecules. Investigators have often resorted to pre-conjugation labeling in these cases.

The use of an acetyl group for protection can improve the MAG_3 labeling method both by simplifying both the synthesis and deprotection/labeling. The ideal protecting group will be stable to indefinite storage of the conjugated molecule and

would hydrolyze under mild conditions only at the point of labeling with technetium-99m. The acetyl group appears to satisfy these requirements. Firstly, the acetyl protected NHS-MAG_3 may be prepared in a one-pot, two-step synthesis not requiring intermediate purification steps. Conjugation to an amine-deriviatized single-stranded DNA was successful.

HPLC analysis of certain MAG_3-DNA complexes suggested the presence of DNA dimers, as evidenced by the appearance of a peak consistent with a DNA dimer. Dimer formation was apparently pH dependent, and was not the result of covalent bonding. To suppress dimerization or other artifacts due to potential hydrogen bonding, the pH of the buffer used to purify and store the radiolabeled DNA was reduced from 7.0 to 5.2 with the result that the peak attributed to dimer formation no longer appeared.

Labeling efficiencies were similar for tartrate-transchelated MAG_3-conjugated DNA relative to that achievable with SHNH-conjugated DNA. In the latter case, 70% labeling efficiency was routinely observed when using tricine as transchelator and specific activities in excess of 100 microCuries/microgram have been obtained (Hnatowich D.J., Winnard P. Jr., Virzi F, Fogarasi M, Sano T, Smith CL, Cantor CR, Rusckowski M., J Nucl Med 36: 2306-2314, 1995). Labeling efficiencies with MAG_3-DNA averaged about 85% with specific activities of about 70 microCuries/micrograms. These values did not increase at 100°C.

In this investigation, an important observation is the different extents to which labeled DNAs to bind to serum proteins. Whereas the technetium-99m label was rapidly bound nonspecifically to serum proteins in 37°C serum when incubated as labeled SHNH-DNA, no evidence of serum protein binding was apparent even after 24 hrs in this medium as labeled MAG_3-DNA (Fig. 4). Rather, low molecular weight peaks were evident in the HPLC radiochromatograms and are almost certainly due to nuclease digestion in serum of the phosphodiester DNAs. In an earlier investigation, we observed similar low molecular weight peaks following serum incubation in the case of a ^{111}In-labeled DNA which, unlike technetium-99m-SHNH-DNA, showed no evidence of nonspecific binding to serum proteins and therefore no protection against nucleases (Hnatowich D.J., Winnard P. Jr., Virzi F, Fogarasi M, Sano T, Smith CL, Cantor CR, Rusckowski M., J Nucl Med 36: 2306-2314, 1995).

It appears that from the Examples above MAG_3 is a useful chelator for DNA since its synthesis has been simplified and the labeling procedures used in this investigation have provided adequate labeling efficiencies and specific activities. Furthermore, unlike the SHNH chelate. the MAG_3 chelate of technetium-99m shows a limited tendency to bind nonspecifically to proteins.

Example 8. PNA Labeling. Two complementary 15-base single-stranded PNAs were synthesized by PerSeptive Biosystems, Framingham, MA. One strand was derivatized with a primary amine on the amino terminus (i.e. 5' equivalent) end via a 17-member ethylene-ether linkage. The complementary sequence was prepared with a biotin group on this end via the same linker. The base sequences were NH_2-$(CH_2)_2O(CH_2)_2OCH_2CONH(CH_2)_2O(CH_2)OCH_2CO$-TGT-ACG-TCA-CAA-CTA-$CONH_2$ and biotin-$(CH_2)_2O(CH_2)_2OCH_2CONH(CH_2)_2O(CH_2)OCH_2CO$-TAG-TTG-TGA-CGT-ACA-$CONH_2$. The melting temperature (i.e., the temperature at which half the base pairs have dissociated) in physiological saline of the duplex was calculated to be 72 °C. The calculated molecular masses were 4336 and 4634 Da respectively and were observed by mass spectrometry to be 4340 and 4635 Da. Purity of both chains was established by reverse phase HPLC (in both cases showing a single peak) and mass spectrometery (showing one predominant peak). The PNAs were lyophylized, stored dry and dissolved when needed in sterile water to a concentration of 4 mg/ml. After solubilization, aliquots of 20-1000 micrograms of PNA were added to plastic vials which were immediately frozen at -20°C for storage. Avidin (Sigma Chemical Co., St. Louis, MO) was purchased and used without further purification. The technetium-99m-pertechnetate was obtained from a ^{99}Mo-technetium-99m radionuclide generator (Dupont, Billerica, MA). Streptavidin-conjugated magnetic polystyrene beads, 1 micron in size (BioMag, PerSeptive Biosystems, Framingham, MA), were stored wet at refrigerator temperatures as recommended by the manufacturer. The capacity of the beads for biotin was reported by the manufacturer to be 1.5 nanogram of biotin per milligram of beads.

The desired volume of the 4 mg/ml water solution of the amine-derivatized single stranded PNA was made 0.36 M $NaHCO_3$, 1.4 M NaCl, and 1.4 milliM DTPA, pH 9.3. The NHS-acetyl-MAG_3 was dissolved in dry DMF at a concentration of 20 milligrams/milliliters. A volume of the DMF solution representing a molar ratio of MAG_3 to PNA of approximately 20:1, was added to the PNA solution during vortexing. The solution (now containing no more than 10% DMF) was incubated at room temperature for 1 hr. The conjugated PNA was purifed over a 0.7 x 30 cm column of P4 (BioRad , Melville, NY) using 0.25 M ammonium acetate, 0.25 mM DTPA, pH 5.2 as eluant. The final PNA concentration was determined by UV absorption at 260 nanometers using an extinction coefficient determined in this laboratory of 33 microliters/micrograms and was usually about 0.3 mg/ml.

The coupled PNA was usually stored frozen at -20°C for no more than one week before use. Generally, 150 micrograms of the conjugated PNA was labeled on each occasion. To the PNA solution (about 300 microliters) was added 2.35 milligrams of Natartrate (Sigma) from a fresh 50 mg/ml solution in 0.5 M ammonium bicarbonate, 0.25 M ammonium acetate, 0.18 M ammonium hydroxide, pH 9.4 buffer, followed by about 5 milliCuries of technetium-99m-pertechnetate generator eluant (20 microliters). Finally, 17 micrograms of tin(II) chloride (Sigma) from a fresh 1 mg/ml solution in 10 mM HCl was quickly added with agitation. The labeled PNA was purified on a 0.7 x 30 cm column of P4 using saline as eluant. The identical labeling procedure was performed on the native, uncoupled PNA as a control.

Each preparation of radiolabeled PNA was analyzed by size exclusion high performance liquid chromatography (HPLC) using a single 30 cm Superose 12 column (Pharmacia, Piscataway NJ) with both in-line radioactivity and UV detection and 0.05 M phosphate, pH 7 eluant. Recovery of radioactivity was routinely determined. Confirmation of labeling was established by HPLC analysis before and after adding the sample to streptavidin-conjugated magnetic beads to which the biotinylated complementary PNA was bound (see below). Loss of radioactivity from solution was due to binding by hybridization of the labeled PNA to the beads.

Labeling efficiencies varied from 30-70% and specific activities as high as 100 microCuries/micrograms of PNA were achieved. Control studies in which the unconjugated PNA was labeled showed less than 1% labeling under identical conditions. The HPLC radiochromatographic profile of the labeled and purified PNAs usually consisted of three distinct peaks, the relative intensities of which varied from preparation to preparation. Figure 5A presents a radiochromatographic profile of one such preparation. That the label was on PNA was confirmed by adding to the labeled PNA solution an excess of complementary PNA. The radiochromatogram resulting from this addition is shown in Fig. 5B and may be compared to the radiochromatogram of the labeled PNA itself (Fig. 5A). A slight shift to higher molecular weight (i.e. earlier fractions on left) has occurred in the case of each peak in the triplet and is the result of PNA-PNA hybridization. Further evidence for labeled PNA is shown in Fig. 5C. In this case, avidin was added to the PNA-PNA duplex to bind the complementary PNA through the biotin moiety. The shift in the radiochromatographic profile is now more pronounced, as expected from the much larger size, and is nearly quantitative. That this shift is not due to nonspecific binding of the label to avidin was established in a repeat study in which avidin was added to the labeled PNA without the prior addition of

complementary PNA. These data show that the radiolabeled PNA hybridizes *in vitro*, and that the biotin moiety functions to bind to streptavidin.

Example 9. Rate of hybridization. Complementary PNA was bound to streptavidin on magnetic beads through its biotin moiety. The suspension of beads was rinsed three times with a washing buffer consisting of 20 mM tris, 2 M sodium chloride, 1 mM EDTA, and 0.1% tween 20 , adjusted to pH 7.0 and six additional times with a 1:1 dilution in water of this buffer. The beads were manipulated for washing by using a magnetic separator (MPC, Dynal, A.S., Lake Success, NY). Following the last wash, the beads were incubated for 30 min with biotinylated complementary PNA at 6 micrograms of PNA per milligrams of beads (i.e. 100 % of saturation) in the washing buffer. The beads were then washed five additional times with the diluted washing buffer.

The rate of hybridization of the labeled PNA to its complement under the conditions of this study was determined at room temperature by adding 1 microgram of labeled PNA to 300 microliters of complementary PNA attached to beads and suspended at a 1 mg/ml concentration in 10 mM tris, 1 M sodium chloride, 0.5 mM EDTA, 0.05% Tween 20, adjusted to pH 7.0 buffer. Samples were removed for analysis periodically over 24 hrs. The beads in each sample were separated magnetically from the solution, washed five times in the washing buffer and counted in a NaI(Tl) well counter. As a control, the identical study was repeated with beads without the complementary PNA.

Figure 6 shows the percentage of labeled PNA bound to complementary PNA on beads as a function of time with early time points separated by 10 min. Under the conditions of this study, hybridization occurs rapidly and is essentially completed within an hour, and mostly completed within the first 10 minutes. The extent of nonspecific binding of labeled PNA to the beads is minimal, as shown by the control study in which identical beads without PNA were used. These data show that PNA molecules form double-stranded DNA complexes rapidly with complementary strands.

Example 10. Serum and Whole Blood Incubations. Labeled PNA was incubated at a concentration of about 5 micrograms/milliliters in fresh 37 °C human serum from two healthy volunteers and in fresh mouse serum. Samples were periodically removed over 24 hrs for HPLC analysis using a 0.1 M sodium phosphate, 0.15 M saline buffer, pH 7.0 eluant. The identity of labeled PNA peaks in the serum incubate was confirmed by HPLC analysis before and after the addition of 200 micrograms of complementary PNA beads to 100 microliters of the serum.

To evaluate whether the labeled PNA accumulates in formed elements, the labeled PNA was also added to fresh human whole blood with EDTA anticoagulant. The whole blood was incubated at 37°C with gentle agitation every 15-20 min. Samples were removed at 1 and 24 hrs and separated by centrifugation. The formed elements (material of cellular origin) were washed three times with 0.1 M PBS, 0.15 M NaCl, pH 7.4, and counted in a NaI(Tl) well counter.

Figure 7 presents radiochromatographic profiles for labeled PNA after 1 and 24 hrs of incubation in 37°C human serum and after 24 hrs in saline. Multiple peaks are again apparent for the labeled PNA in Fig. 7A. In serum, minimal binding of the label to serum proteins is apparent (Figs. 7B and 7C). The radioactivity ratios among the triplet PNA peaks have been consistently observed to changes in serum in favor of the peak eluting in fraction 83. One peak, eluting in fraction 105 in the Figure, is probably the result of catabolism. These general features were also observed during incubations in mouse serum and in human serum. A change in the radioactivity profile also occurs during incubation in room temperature saline (Fig. 7D)

To help identify radioactive peaks in serum due to labeled PNA, complementary PNA bound to streptavidin beads were added to a 1 hr serum sample and the sample reanalyzed after filtration to remove the beads. Figure 8 shows the radiochromatogram of the labeled PNA initially in saline (Fig. 8A) and after 1.5 hrs in serum before (Fig. 8B) and after (Fig. 8C) extraction of labeled PNA. Only the serum bound radioactivity remains after extraction. As a control against nonspecific binding, the identical serum sample was extracted under identical conditions except with beads without complementary PNA. As shown (Fig. 8D) the radiochromatogram is in this case unchanged.

Under the conditions of incubation of labeled PNA in whole blood described above, radioactivity bound to formed elements was 1.0% and 2.7% at 1 and 24 hrs, respectively. This data indicates that radionuclide-MAG_3-PNA is very stable, and that there is little non-specific binding to serum or material of cellular origin. These data indicate that the radionuclide-MAG_3-PNA composition is very stable in serum, that little of the radioactivity binds to serum proteins or is catabolized, and that little is associated with cellular material or cellular debris.

Example 11. Homogenate and Urine Analysis Studies. Normal CD-1 male mice (Charles River, Wilmington, MA) were injected via the tail vein with 0.1 milliliters of 0.15 M saline containing about 5-10 micrograms (about 100 microCuries) of labeled PNA, and samples of urine were obtained at 1 hr post administration. The animals were

sacrificed by cervical dislocation at 2.5 hrs and the kidneys removed. Homogenates were prepared in a 15 milliliters tissue grinder (Dounce, Wheaton, Millville, NJ) in ice-cold 0.2 M sodium acetate buffer, pH 5. After grinding, samples were sonicated for 0.5-1 min at 300 watts on ice and then centrifuged at 3500 rpm at 4 °C for 15 min. The pellet and supernatant were counted separately and aliquots of the supernatant were also analyzed by HPLC.

Mouse urine collected 1 hr post administration was analyzed by HPLC before and after adding the complementary PNA beads to establish the extent to which radioactivity in urine was technetium-99m-MAG_3-PNA. Control studies consisted of the identical assay in which beads without PNA were added to the urine sample.

Figure 9A presents HPLC radiochromatograms of labeled PNA administered to normal mice. The Figure also shows the results of analyzing the serum (Fig. 9B) and urine samples (Fig. 9D) obtained at 2.5 hrs post administration. The serum sample shows only a single radiolabeled serum peak while the urine sample shows one of the labeled PNA peaks. Figure 9E is a repeat radiochromatogram (now presented on an expanded scale) obtained by analyzing the urine after the addition of complementary PNA beads. Virtually all radioactivity has been removed showing that the label in urine is present as labeled PNA. Figure 9C presents a radiochromatogram of the soluble fraction from the homogenate of a kidney obtained at this time showing labeled PNA and higher molecular weight, presumably labeled proteins. However, this analysis considers only about 20 % of the radiolabel in the kidney since the remainder appeared in the insoluble pellet.

Example 12. Animal Biodistribution and Imaging Studies. Biodistributions of technetium-99m-MAG_3-PNA were evaluated in normal CD-1 male mice. Each animal was administered by tail vein 0.1 milliliters of 0.15 M saline containing 5 micrograms (about 100 microCuries) of technetium-99m-MAG_3-PNA. Whole body activity was determined by repeatedly placing each animal momentarily in a dose calibrator. The anesthetized animals were sacrificed by spinal dislocation at 2.5 and 24 hrs post administration. Samples of organs were rinsed in cold saline and were weighed before being counted in a NaI(Tl) well counter along with a blood sample and an aliquot of the injectate. The biodistributions were reported as percent of the administered radioactivity per gram of tissue.

In a separate study, three male CD-1 mice were each injected intramuscularly in their left thighs with 150 milliliters of saline containing 1.0 milligrams of PNA-coupled beads. An equivalent quantity of beads without PNA was injected into their contralateral

thighs. Immediately thereafter, each animal received an intraperitoneal injection of 50-55 micrograms (about 1 milliCurie) of labeled PNA. Animals were imaged simultaneously on an Elscint APEX 409M portable gamma camera by resting the nembutal-anesthetized animals on the face of the upright collimator. Animals were imaged five times between 2 and 23 hrs post administration of the labeled PNA. At sacrifice, both whole thighs were excised for counting in a NaI(Tl) well counter. Regions of interest were drawn about the thighs and whole body in each image to obtain an estimate of the counts therein. Based on the counts in each image, the well counter counts of the thigh and the injected activity, the percent of the injected dosage in each thigh at each time point was estimated. The left thigh/whole body radioactivity ratios were calculated without correction simply from the counts in each image.

Figure 10 shows the whole body radioactivity plotted separately for each of ten mice receiving 5 micrograms each of labeled PNA. The half time of clearance is approximately 2 hrs.

Table 1 presents the biodistribution results obtained at 2.5 and 24 hrs post administration of the radiolabeled PNA. The results reflect the rapid clearance of the label shown in Fig. 10; the highest radioactive content is only 1.45% of the injected dose per gram (ID/gm) at 2.5 hrs and 0.07 % ID/gm at 24 hrs (in both cases in kidneys).

Three animals received identical administrations of beads subcutaneously in both thighs prior to the IP administration of the labeled PNA. In all cases, only the beads in the left thigh contained the complementary PNA. Figure 11 presents the mean percentage of injected radioactivity in the left and right thighs along with the left/right thigh radioactivity ratios at each time point. The left thigh/whole body ratio rose from 0.05 at 2 hours to 0.09 at 23 hours post injection. As is evident from the Figure, the left/right thigh radioactivity ratio rose from 2.7 to 5.8 during this period. A composite whole body anterior-posterior image of the three animals imaged simultaneously at 23 hrs post administration shows radioactivity essentially only in kidneys, bladder, and the left thigh. These data and the left/right thigh radioactivity demonstrate *in vivo* hybridization of PNA-MAG_3-radionuclide to its complement.

Table 1

Organ	2.5 hrs	24 hrs
Liver	0.19(0.08)	0.010(0.002)
Heart	0.05(0.03)	0.001(0.002)
Kidneys	1.45(0.88)	0.065(0.017)
Lung	0.11(0.05)	0.002(0.001)
Stomach	1.30(0.82)	0.050(0.027)
Spleen	0.05(0.02)	0.000(0.001)
Muscle	0.06(0.04)	0.001(0.002)
Intestine	0.18(0.11)	0.007(0.004)
Blood	0.17(0.07)	0.000(0.000)

Table 1 shows the mean biodistribution (in percentage injected dose/milligrams of tissue) obtained in normal mice at times of 2.5 hrs and 24 hrs post intraperitoneal administration of technetium-99m-labeled-MAG_3-PNA. The data are the mean of values from five experimental animals, with standard deviation in parenthesis.

For use in radiopharmaceutical applications, oligomers must possess certain essential properties. Since diagnostic applications require only tracer quantities of drug, toxicity is unlikely to be an issue. Among other considerations, suitable stability of the oligomer *in vivo* is also essential. In addition, the pharmacokinetic properties must be suitable for the intended application. For example, the oligomer should clear through the kidneys in a time frame consistent with the application to provide a favorable target/nontarget ratio. For use as radiopharmaceuticals, it must be possible to radiolabel with imagable radionuclides such as technetium-99m and that the label be suitably stable *in vivo*. Finally, the labeled oligomer must be capable of hybridization *in vivo* with its complement in the target.

Although the *in vivo* properties were influenc- ·o some extent by the method of radiolabeling, the phosphodiester DNA was judged t egraded by nucleases too rapidly for most applications. The phosphorothioate i although stable towards nuclease digestion, showed a high affinity for serum an ssue proteins. As a consequence, background radioactivity in liver and other tissues was present at high levels.

Peptide nucleic acids are synthetic oligomers in which the sugar and phosphate backbone of oligonucleotides have been replaced with a polyamide linkage. Not only

does this substitution provide an oligomer resistant to nuclease and protease attack, but the absence of charge improves the binding affinity of PNA-DNA heteroduplexes.

In these examples, radiolabeling with technetium-99m was achieved by means of an acetyl-protected MAG_3 chelator. This labeling strategy was developed to avoid "nonspecific" serum protein binding observed for DNA labeled using a hydrazino nicotinamide (SHNH) chelator. Similar properties for technetium-99m *in vitro* and *in vivo* are seen in animals when labeled to two IgG antibodies by MAG_3 and SHNH chelators (data not shown). Using MAG_3, respectable labeling efficiencies and specific activities were achieved for PNA. Furthermore, the stability of the label in 37°C serum was acceptable with minimal activity present on either higher or lower molecular weight species (Fig. 7).

Although a UV profile of PNA showed a single peak by HPLC analysis (data not presented), the radioactivity profiles always consisted of two to three distinct peaks with only the earliest eluting with UV absorbency. Furthermore, the HPLC radioactivity profile varied somewhat from preparation to preparation but showed a consistent and profound shift to the third (i.e. last) peak on incubation in saline and, especially, in serum (Figs. 7, 8). That all three peaks were radiolabeled PNA was established by demonstrating that each shifted to higher molecular weight upon addition of the complementary PNA (Fig. 5). Thus, the radionuclide is maintained in the complex with PNA.

It has been reported that PNA undergoes negligible transport across cell membranes (Pardrige WM., Boado RJ., Kang Y-S. *Proc Natl Acad Sci USA 92*: 5592-5596; et.al. 1995). Support for this property of technetium-99m-MAG_3-PNA may be found in the negligible accumulation of radiolabel in formed elements following incubation of labeled PNA in whole blood.

These examples show that the properties of PNA labeled according to the methods used herein are suitable for imaging studies *in vivo*. Apart from the stability of the label discussed above, the pharmacokinetic properties are favorable. Whole body radioactivity in mice following intravenous administration showed a rapid decrease (Fig. 10). The biodistribution studies in normal mice (Table 1) also showed this rapid decrease. At 2.5 hrs, the highest level of radioactivity was in kidneys at only 1.45 % ID/gm. At 24 hrs, radioactivity in several tissues were below detectability. These results are in sharp contrast to that observed in this laboratory for technetium-99m-labeled phosphodiester and phosphorothioate DNAs of about the same chain length where tissue radioactivity levels (except for stomach) at 4 hrs post-administration were about 2-10 times higher for the phosphodiester DNA and 10-400 times higher for the

phosphorothioate DNA incorporated here by reference. As shown (Fig. 6), hybridization under one set of conditions is more than 50% complete in less than 10 min.

A test of suitability for medical use of oligomers may be *in vivo* hybridization. Only in the left thigh were the implanted beads first bound with the complementary PNA. Following intraperitoneal administration of radiolabeled PNA, increased accumulation of label occurred in the left thigh due to hybridization, with the left/right radioactivity ratio increasing with time between 2 and 23 hrs. Apart from radioactivity in the left thigh, the whole body image show radioactivity only in bladder and kidneys.

These data demonstrate that single stranded PNA may be radiolabeled with technetium-99m using the MAG_3 chelator. Importantly, when administered to mice, radiolabeled PNA can be made to hybridize *in vivo*.

Example 13. Pretargeting and Targeting with PNA-MAG_3-^{99m}Tc in a Mouse Model Infection. A strategy for pretargeting for imaging and therapy of tumors and infections in subjects is to use single stranded peptide nucleic acid (PNA) of a given sequence as a pretargeting first administration, followed by administration of complementary sequence single stranded PNA-MAG_3-^{99m}Tc of the subject invention. In this strategy, affinity of the pre-targeting first administration for the second administered therapeutic agent of the invention causes that agent to be localized to the correct target site. This strategy was applied to a mouse model for infection and a mouse tumor model by in which the "pretargeted" material circulates in the study animal by passive diffusion, and localization of radiolabeled complementary PNA is targeted by *in vivo* hybridization. The pretargeting material chosen for this model was PNA-streptavidin, prepared by addition of streptavidin to biotin-conjugated PNA.

The complementary strand PNA, derivatized with a primary amine, was conjugated with acetyl S-protected NHS-MAG_3 and radiolabeled with ^{99m}Tc as described in Examples, *supra*. Sufficient time provided between the two administrations so that the earlier administered composition localizes to the target, and non-localized material can be cleared from circulation and normal tissues.

In the present application, the term "pre-targeting" molecule, agent, composition or compound refers to a first-administered non-radioactive molecule. The term "targeting" molecule, agent, composition or compound used in the context of pre-targeting strategies, refers here to a second administered composition in which a targeting moiety (e.g., a member of a specific binding pair, e.g., a nucleic acid sequence, an antibody, a biotin compound, or the like), derivatized with a chelator which complexes a radionuclide, is a ligand of (e.g., can bind to) the first administered agent.

The targeting agent delivers the radioactive agent specifically, e.g., to a tumor or infectious agent or infected cell. The strategy localizes the activity of the radioactivity to the targeted tumor or infection by virtue of the interaction between the targeting agent and the pre-targeting agent. The targeting agents described herein, for example, PNA-MAG_3-^{99m}Tc having a given base sequence, are applicable to wide variety of pretargeting compositions, e.g., which comprise a PNA sequence complementary to a targeting agent but differ in affinity for, e.g., a particular tumor type or infectious disease antigen.

The design of pre-targeting moieties and useful targeting moieties with radionuclides is well known in the art (Hnatowich, D.J., Virzi F., Rusckowski, M., *J Nucl Med 28:*1294-1302, 1987; Paganelli, G., Magnani, P., Zito, F., et al., *Can Res 51*: 5960-5966, 1991). Biotin compounds and their ligand proteins, avidin and streptavidin, can be employed in the invention (Hnatowich D.J., *Nucl Med Comm. 15*: 575-577, 1994; Rosebrough, S.F., *J Pharmacol Exp Ther 265*: 408-415, 1993; and Paganelli, G., Malcovati, M., Fazio, F., *Nucl Med Comm 12*: 211-234,1991). More preferred compositions of the invention for pretargeting exploit the high affinities of complementary nucleic acid strands for each other. For example, useful molecules can comprise a nucleic acid covalently linked to a protein ligand, or antibody, specific for a tumor or infection, such that the antibody is a pre-targeting agent; then the complementary sequence of nucleic acid, covalently linked to a chelator and a radionuclide, comprises the targeting agent as defined herein. Affinities of single-stranded DNA for the complementary strand can approach that of biotin to streptavidin (Egholm, M., Kim, S.K., Norden, B., Nielsen, P.K., *Nature 365*: *566-568.* 1993). Pretargeting with a 15-base single-stranded DNA, covalently conjugated to an antitumor antibody, was used as the first administration in an *in vitro* two-step strategy employing a radioiodine-labeled complementary single-stranded DNA as second step (Bos, E.S., Kuijpers, H.A., Meesters-Winters, M., et al., *Can Res 54*: 3478-3486, 1994). As shown in Examples 8-12 above, PNA possesses properties suitable for *in vivo* targeting use, and is most preferred as an affinity component for binding the pretargeting and targeting chelator-radionuclide agents to each other.

Materials and methods for Examples 13 and 14 are given in previous Examples (*supra*), and the sequence and structure of the 15-base PNA strands is given in Example 8. The strand for pretargeting is shown in Example 8 derivatized with a biotin group on the amino terminus end (i.e. 5' equivalent) through a 17-member ethylene-ether linkage. The complementary 15 base sequence, used here for radiolabeling through covalent linkage to MAG_3, contains a primary amine group on this end from the same 17-

member linker, which, as described above, was used to covalently link the chelator. The expected molecular masses, measures of purity, preparation of aliquots, reconstitution, and procedures for linkage and transchelation of radionuclide are described above. In preliminary studies with infected animals, the dosage of PNA-streptavidin was varied from 60 to 150 micrograms and the dosage of labeled PNA varied from one to 100 micrograms. Further, the interval between the two administrations was varied from 4 to 17 hrs and the period from the second administration to sacrifice was varied from 3 to 18 hrs (data not presented). On the basis of the results obtained, the optimum dosages and timing for the infected and tumored animal studies was estimated.

The mouse model for an infectious disease was prepared with a clinical isolate of *Escherichia coli* ATCC #25,922 (American Type Culture Collection, Rockville, MD.). The culture was grown in peptone yeast extract-glucose broth (Becton-Dickinson, St. Louis MO.) at 37°C to a density of 10^8 organisms/ml, and was stored at 4°C. Male CD-1 mice (Charles River) weighing approximately 25 g were anesthetized by inhalation of metofane (Pitman-Moore, Inc., Washington Crossing, NJ.) and were inoculated subcutaneously in the posterior left thigh with 0.1 ml of the bacterial culture. Swelling was observed after 16 hr, at which time 150 micrograms of PNA-streptavidin was administered intravenously through a tail vein. After 5 hr, each animal received an intraperitoneal dose of 50 micrograms (200 microCuries) of radiolabeled complementary PNA. Control infected animals received the labeled PNA only, and not the earlier-administered complementary PNA-streptavidin. Animal were imaged at 4 hr after administration of the radioactivity and killed immediately thereafter by cervical dislocation.

For imaging, animals were anesthetized with nembutal (Abbott Laboratories, North Chicago, IL) using an intraperitoneal dose of 1.0 milligrams per 25 g mouse, administered in 0.1 ml of saline. The animals were then positioned directly on the collimator. Typically, several animals were imaged simultaneously at 4 hrs after administration of the label. Scintigrams were acquired using a portable large field of view scintillation camera (Elscint, Hackensack, NJ) equipped with a parallel-hole, medium energy collimator and an Elscint APEX F1 computer. Following sacrifice, animals were dissected to provide samples of tissue for counting in a well NaI(Tl) counter, and results are expressed as percent of injected dose per gram of tissue. The entire infected left leg and for comparison, the entire right leg, were each removed for counting.

Table 2 presents biodistribution results for the model infection mice, at 4 hrs post intraperitoneal administration of radio-labeled complementary PNA and 9 hrs after

intravenous administration of the PNA-streptavidin. With the exception of the kidneys, stomach, spleen, and intestines, tissue radioactivity levels were found to be significantly higher in the study animals compared to control animals not receiving the PNA-streptavidin prior to the targeting composition. This increase is related to administration of pretargeting PNA-streptavidin in concentrations sufficient to bind the subsequently administered radiolabeled PNA complement.

Mean infected to normal thigh ratios from the results presented in Table 2 are 3.46 (s.d. 0.55) and 1.67 (s. d. 0.17) for study and control animals, respectively. The difference is significant at P equals 0.0001. Thus, this ratio was effectively doubled by the pretargeting administration of the PNA-streptavidin. Moreover, the mean uptake in the infected thighs of animals receiving PNA-streptavidin was increased by a factor of four over the infected thighs of control animals not receiving PNA-streptavidin. Finally, the infected thigh to tissue radioactivity ratios for all tissues were higher for the study animals compared to the control animals.

Table 2

Tissue	Study Animals	Control Animals	P value
Liver	1.17 (0.31)*	0.41 (0.04)	0.0006
Heart	0.44 (0.16)	0.13 (0.02)	0.003
Kidneys	6.03 (2.53)	4.11 (1.62)	NS**
Lung	0.73 (0.40)	0.22 (0.03)	0.02
Stomach	1.36 (0.49)	1.06 (0.31)	NS
Spleen	1.32 (0.94)	0.88 (0.08)	NS
Small Intestines	0.76 (0.70)	1.04 (1.10)	NS
Infected Thigh	0.67 (0.22)	0.16 (0.03)	0.0009
Normal Thigh	0.19 (0.04)	0.10 (0.02)	0.001
Blood	1.19 (0.29)	0.53 (0.08)	0.001

Biodistribution of ^{99m}Tc-PNA 4 hrs after administration to mice with left thigh infections. Study animals received complementary PNA-streptavidin 9 hrs earlier; control animals did not receive PNA-streptavidin. P values are also listed (no. of animals = 5)

*Percent injected dose per gram; one standard deviation of the mean in parenthesis.

**Not Significant

Whole body anterior images of control animals showed activity in bladder (and possibly kidneys) with faint accumulation in the left thigh. The study animals showed similar activity in bladder, increased activity in liver and, in particular, increased uptake in the infected thigh. Increased localization of radiolabel was achieved in animals receiving both injectates of pre-targeting and targeting compositions, in comparison to the control animals receiving only the radiolabeled PNA as the targeting material. These data show that PNA linked to a protein and PNA-MAG_3-^{99m}Tc are suitable compositions for pretargeting and targeting, respectively.

Example 14. Pretargeting and Targeting with PNA-MAG_3-^{99m}Tc in a Mouse Tumor Model. The LS174T tumor was obtained from American Type Culture Collection and was grown in minimal essential medium (Gibco, Grand Island, NY). Cells were removed from the culture flask by trypsinization and then washed in the culture medium. Swiss male nu/nu mice (Taconic Labs., Germantown, NY) were injected subcutaneously in the flank of the left thigh with 10^6 cells in 0.1 ml of medium. Two weeks later, when tumors were approximately 0.6 cm in diameter, each study animal received 150 micrograms of PNA-streptavidin, and 5 hrs later all animals received 50 micrograms of PNA-MAG_3-^{99m}Tc of complementary base sequence. Animals were imaged 3 to 4 hrs later, sacrificed, and dissected as in Example 13.

Table 3 presents biodistribution results 3 to 4 hrs after intraperitoneal administration of labeled PNA and 9 hrs after intravenous administration of pretargeted PNA-streptavidin in mice implanted in the left thigh with the LS174T tumor cells. Results are presented for both the study and control animals which did not receive the administration of complementary PNA-streptavidin. As in Example 13, tissue radioactivity levels were found to be significantly higher in most tissues in study animals compared to control animals. This increase is related to the presence in study mice of PNA-streptavidin in concentrations sufficient to bind and retain the complementary radiolabeled PNA of the second injection.

The mean tumor to normal thigh ratios from the results presented in Table 3 are 1.70 (s.d. 0.15) and 1.28 (s. d. 0.16), for study and control animals, respectively. The difference is significant at $P = 0.003$. As in the study of the infected mice in Example 13, prior administration of PNA-streptavidin significantly improved the PNA-MAG_3-^{99m}Tc uptake ratio. Moreover, the mean uptake in thighs with tumors was increased by a factor of three over that in thighs with tumors of control animals not receiving PNA-streptavidin. Finally, the tumored thigh to tissue radioactivity ratios were higher for study animals compared to control animals in all tissues except liver and lung. The

whole body anterior images of the control animals showed whole-body activity with faint accumulation in the tumor on the left. The study animals showed similar activity in the whole body, and increased uptake in the tumored thigh compared to the control thigh. These data show that complementary PNA strands are suitable affinity ligands as an alternative to (strept)avidin and biotin for design of pretargeting and targeting compositions.

For pretargeting with PNA and other nucleic acid oligomers, selection of the length of the sequence and the base sequences of the PNA compositions to be radiolabeled will be routine for the skilled artisan. These factors, and use of a clearing agent, influence the pharmacokinetics of the pretargeting composition, and the pharmacokinetics of the radiolabeled composition *in vivo* for pretargeting binding and non-specific background, and hence the therapeutic index.

In both the infection model (Example 13) and the tumor models (Example 14), accumulation of label in most tissues was significantly increased by prior injection of PNA-streptavidin (Tables 2 and 3, respectively), indicating *in vivo* hybridization in tissue of PNA-MAG_3-^{99m}Tc with its complementary PNA. The pretargeting PNA-biotin-streptavidin is not an affinity ligand for the infection or the tumor, and localized to sites by passive diffusion. It is rather a model affinity molecule for the radiolabeled PNA-MAG_3-^{99m}Tc. Significant retention of radiolabel in the pre-treated study animals found in Examples 13 and 14 shows that single-stranded PNA properties of stability, high binding affinity to the complement, and rapid clearance *in vivo*, make these oligomer-chelator-radionuclides useful novel agents and targeting compositions in a pretargeting drug strategy.

Table 3

Tissue	Study Animals	Control Animals	P value
Liver	1.19 (0.21)*	0.32 (0.06)	0.0001
Heart	0.23 (0.05)	0.10 (0.03)	0.003
Kidneys	5.25 (2.11)	4.67 (1.74)	NS**
Lung	0.65 (0.19)	0.19 (0.05)	0.003
Stomach	3.46 (3.83)	1.39 (0.44)	NS
Spleen	1.00 (0.45)	0.77 (0.29)	NS
S. Intestines	1.36 (1.43)	0.52 (0.57)	NS
Tumored Thigh	0.29 (0.07)	0.10 (0.02)	0.0009
Normal Thigh	0.17 (0.04)	0.08 (0.02)	0.006
Blood	0.69 (0.17)	0.43 (0.05)	0.02

Biodistribution of ^{99m}Tc-PNA 3 to 4 hrs after administration to mice with left thigh tumors. Study animals received complementary PNA-streptavidin 9 hrs earlier; control animals did not receive PNA-streptavidin. P values are also listed (no. of animals 4,5).

*Percent injected dose per gram, one standard deviation of the mean in parenthesis.
**Not Significant

Example 15. Synthesis of a library of novel amidothiol chelators for radionuclides, and conjugation to biocytin. A combinatorial chemistry approach to identify one or more amidothiol bifunctional chelators with properties complementary or superior to that of MAG_3 is accomplished by synthesis of a library of approximately 70 chelators using a novel synthetic route. Each is labeled with technetium-99m (^{99m}Tc) and useful properties (charge, lipophilicity and maximum specific activity) of each labeled chelator is detemined. For each chelator, the NHS ester is prepared for conjugation to the biotin compound biocytin. Each biotin-conjugated chelator radiolabeled with ^{99m}Tc is tested for stability of label in saline, in serum, and in cysteine, to determine stability of radiolabel and nonspecific affinity for serum proteins. Chelators with useful properties are characterized by elemental analysis and NMR to establish structures. Successful chelators are conjugated to amine-derivatized polymers, including antibodies and at least one polypeptide (EGF) to evaluate the influence of the novel structure on biodistribution in normal mice in comparison with MAG_3. Each free chelator radiolabeled with ^{99m}Tc

is evaluated for biodistribution in normal mice, and each ^{99m}Tc-biotin is evaluated for use in (strept) avidin/biotin pretargeting studies. Chelation to ^{188}Re is also determined.

A comparison of the ^{99m}Tc complexes of SHNH- and MAG_3-conjugated human serum albumin has shown that SHNH more stable than MAG_3 with respect to cysteine transchelation (Verbke K., Hjelstuden O., Debrock E., Cleynhens B., De Roo M. Verbruggen A. *Nucl Med Comm* Vol. 16:942-957, 1995). Increased instability of ^{99m}Tc towards cysteine challenge when radiolabeled to the Sandoz and C110 antibodies conjugated to MAG_3 compared to each antibody conjugated to SHNH is shown in Figure 12. Complexes were incubated for 1 hr at 37°C with increasing cysteine. The stabilities are similar at molar ratios of less than about 50:1, and with increasing cysteine the relative instability of MAG_3-chelated ^{99m}Tc in comparison to the SHNH-chelate is seen.

Biodistribution results in normal mice injected with labeled C110 antibody conjugated to each of MAG_3-^{99m}Tc and SHNH-^{99m}Tc are shown in Table 4. Radioactivity in certain tissues (liver, spleen, blood) are elevated in the SHNH case presumably because of increased stability toward *in situ* cysteine transchelation and clearance of labeled cysteine. Similar data were observed with of the Sandoz antibody. Thus, chelators having improved properties were sought.

Table 4

Organ	MAG_3-C110	SHNH-C110	p value
Liver	1.87 (0.35)*	5.63 (1.0)	0.001
Heart	1.00 (0.35)	1.85 (0.44)	0.05
Kidneys	2.83 (0.40)	2.46 (0.24)	0.24
Stomach	0.43 (0.15)	0.71 (0.15)	0.10
Spleen	0.74 (0.16)	1.92 (0.32)	0.003
Muscle	0.73 (0.43)	1.69 (0.94)	0.15
Intestines	0.43 (0.09)	0.75 (0.15)	0.014
Blood	5.17 (0.90)	7.02 (0.63)	0.03

Table 4 shows whole body distribution of ^{99m}Tc at 24 hrs after intravenous administration of C110 IgG antibody labeled with chelates of each of MAG_3 and SHNH. Student's t-test p values are presented.

*Percent injected dose per gram, one standard deviation in parentheses (sample size: 5).

The novel synthesis of MAG_3 in Example 2 *supra*, is the basis for synthesis of novel chelators, which may be summarized as reaction over 15 min at room temperature of triglycine with the NHS ester of S-acetylthioglycolic acid (SATA), in which SATA provides facile acetyl protection for the sulfur. Further, the NHS ester of MAG_3 is readily prepared in the second step by reacting, also at room temperature, free NHS (generated *in situ* or added) with the MAG_3 carboxylate intermediate via dicyclohexylcarbodiimide. Acetyl protection is suitable as the protecting group for both free or polymer-conjugated forms of the chelator. The acetyl protected MAG_3 was found to be stable to long term storage and sulfur deprotection conveniently occurs under the mild conditions of the radiolabeling step. Previous bifunctional amidothiol chelators in the art are not NHS esters and therefore may not be suitable for this well-characterized method of conjugation to amines. As shown in *supra*, acetyl-MAG_3 can be conjugated to DNA, peptide nucleic acids (PNA), peptides and antibodies.

The method of synthesis of acetyl-MAG_3 presented herein can be applied to the synthesis of chelators based on commercially available oligopeptides, e.g., tripeptides. Analytical methods, as described above, are used to ascertain successful reactions, verify chemical identity of product, and to measure activities, and are applied to each novel chelator. A sample of the NHS ester of each chelator is conjugated to biocytin, a biotin compound. Conjugated biocytin derivatives of the chelators are prepared (Figs.13, 14 and 15) and radiolabeled by methods of Examples above. The library of biotin-conjugated chelators is tested for useful properties such as lipophilicity, chelation properties, suitability for ^{99m}Tc pretargeting studies with streptavidin, avidin, or equivalent biotin-binding derivatives of streptavidin or avidin, and the like. Also, biocytin-conjugated chelators have a number of possible therapeutic applications to pretargeting and targeting development of compositions for tumors and infectious diseases.

The structures of three N_2S_2 chelators are shown in Fig. 14, and the synthesis of NHS esters N_2S_2-1 and corresponding biocytin conjugates is shown in Fig. 14, and synthesis of N_2S_2-2 and -3 and corresponding biocytin conjugates is shown in Fig. 15. The three were selected on the basis of properties predicted for each when radiolabeled. For example, N_2S_2-2 should bind ^{99m}Tc by chelation involving two five membered rings and one six membered ring, in contrast to both the other structures which should have three 5-membered rings, differences that might affect stabilities of the labeled complex. Furthermore, N_2S_2-3 is unique among these in its lack of stereoisomerism. Bifunctional N_2S_2 chelators with a rigid aromatic backbone have been successfully prepared and radiolabeled (Liu S. *et al.*, eds., *Technetium and Rhenium in Chemistry and*

Nuclear Medicine, SGEditoriali, Padova, Italy, pp. 383-393, 1995). All three structures should form five-coordinate square pyramidal chelates with an overall -1 charge (Notwitnik, D.P. and S.S. Jurisson, in The Chemistry of Technetium in Medicine Ed. J. Steigman and W. C. Eckelman, *Nat. Acad. Press*, Washington, pp. 111-131, 1992).

The three diaminocarboxylates (1,2 diaminoethane-1-carboxylate; 1,3-diaminopropane,1 carboxylate; and 3, 4 diaminobenzoic acid, respectively) were obtained from Sigma (St. Louis, MO) and reacted with SATA to provide the three respective N_2S_2 chelators as shown in Figs. 14 and 15. Conditions of synthesis of the free chelators were as follows: 0.5 mmoles of the diaminocarboxylic acid was dissolved in 0.5 ml of a NaOH solution at pH 8.8. To this was added dropwise with rapid stirring 1.0 mmol SATA (Sigma) in 0.2 ml dry DMF. The reaction was stirred at room temperature for 15 min, solvent was evaporated under vacuum, and the reaction mixture was dissolved in 0.6 ml of dry DMF. Without purification, a sample of the reaction mixture was subjected to radiolabeling with ^{99m}Tc by two separate procedures, one by direct stannous ion reduction and the other by glucoheptonate transchelation. Direct labeling was accomplished by adding 10 micrograms (about 10 microliters) of the chelator in DMF to 80 microliters of 0.2 M phosphate buffer, pH 8.0. To this was added 200 microCuries of ^{99m}Tc-pertechnetate (2 microliters) and 2 microliters of a fresh 1 mg/ml solution of $SnCl_2.2H_20$ solution in 10 mM HCl. Incubation was at room temperature for 30 min. Radiochemical purity was estimated by Sep-Pak (Millipore) chromatography in which the C-18 column was first equilibrated in 0.001 M HCl solution and, after adding the sample, eluted with this solution. As in the case of ^{99m}Tc-MAG_3, radiolabeled pertechnetate appears in this fraction. Thereafter, the column was eluted with 50% ethanol to obtain the labeled chelator. Depending upon the chelator, between 40-100% of the radioactivity was present in the ethanol solution.

The chelators were also radiolabeled by transchelation from glucoheptonate. In this case the labeling procedure was essentially identical except that 9.4 microliters of a 50 mg/ml solution of fresh sodium glucoheptonate (Sigma) in 1 M $NaHCO_3$, 0.25 mM NH_4Acetate, pH 9 was added prior to the addition of pertechnetate. As determined by Sep-Pak analysis, labeling efficiency varied between 30 and 90% depending upon chelator.

The synthesis of the NHS esters involved adding to 0.6 ml of the DMF chelator solution dicyclohexycarbodiimide (DCC 0.5 mmol) in 0.2 ml of dry DMF and stirring the reaction at room temperature overnight. Most of the dicyclohexylurea was removed by filtration before the solvent was evaporated to dryness, and the NHS ester was then crystallized in 2-propanol.

The general procedure for the preparation of the biotin conjugate was the following: to 2.7 micromoles of biocytin in 200 microliters of 0.2 M phosphate buffer, pH 9.0, was added 13.4 micromole of the bifunctional chelator in 40 microliters dry DMF with vigorous shaking. The reaction was allowed to proceed at room temperature for 30 min. The resulting biocytin-amide conjugate was then evaluated without purification.

Radiolabeling of the biotin conjugates was performed in the sub-microgram scale, and only indirect labeling with glucoheptonate was used. To 10 microliters (about 0.4 ug) of each biotin conjugate in 20% DMF in 0.2 M phosphate buffer, pH 9, was added 2 microliters of the 50 mg/ml solution of fresh sodium glucoheptonate in 1 M $NaHCO_3$, 0.25 mM NH_4Acetate, pH 9 followed by 6 microliters (600 microCuries) of pertechnetate and 2 microliters of the fresh 1 mg/ml solution of $SnCl_2.2H_20$. The reaction was incubated was for 30 min at room temperature, and radiochemical purity was obtained by Sep-Pak analysis, *supra*.

Prior to preparing a library of N_3S chelators from tripeptides shown in Table 5, a preliminary study was performed with the mercapto tripeptide phenylanine-glycine-glycine tripeptide as a representative lipophilic tripeptide, as follows. For synthesis, 0.018 mmoles (5 mg) of phe-gly-gly tripeptide in 68 microliters of 0.23 M NaOH was added to 145 microliters of DMF. While stirring at room temperature, 0.027 mmoles (6.2 mg) of SATA in 23 microliters of DMF was added dropwise. Progress of the reaction was monitored by SG-TLC (Silica G-254) using acetonitrile as solvent. After completion at one hr, the reaction mixture was evaporated to dryness under vacuum at 50°C and the dried residue dissolved in 200 microliters of DMF. To this was added 0.018 mmole (2.3 mg) of NHS in 40 microliters DMF and 0.02 mmole DCC in 100 microliters DMF and the mixture was stirred for 3.5 hrs at room temperature. The reaction was again monitored by SG-TLC using acetonitrile/triethylamine 50/1 (v/v) as solvent. The dicylohexylurea precipitate was removed by filtration, and the yield estimated by TLC was about 50%. The NHS-phenyl-MAG_2 was used immediately without purification.

The free chelator was radiolabeled by adding, in order, the NHS-phenyl-MAG_2, bicarbonate buffer, pH 9.3, sodium tartrate, ^{99m}Tc-pertechnetate eluant and stannous ion. After incubating the reaction for 15 min at room temperature, the labeling yield was determined to be 90% by Sep-Pak analysis.

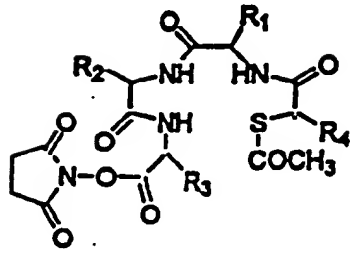
Conjugation of the NHS-phenyl-MAG_2 to biocytin was accomplished by adding 2.4 micromole (1 mg) of biocytin in 300 microliters of 0.5 M bicarbonate buffer, pH 8.9, to 5.3 micromole (about 2.5 mg) of the phenyl-MAG_2 in 200 microliters DMF and the

mixture stirred for 30 min. The phenyl-MAG_2-biotin was stored in the freezer and used without purification.

Radiolabeling of the biotin conjugate was accomplished by adding in order: the conjugate in DMF/bicarbonate buffer, pH 9.3, sodium tartrate, ^{99m}Tc-pertechnetate eluant and stannous ion. After incubating the reaction for 15 min at room temperature, the labeling yield was determined to be 93% by Sep-Pak analysis. Analysis by HPLC (Superdex) showed three radioactivity peaks at retention times of 45, 51 and 63 min. The main UV peak was at 45 min.

That the ^{99m}Tc label was on phenyl-MAG_2-biotin was confirmed by determining binding to an excess of avidin and incubating at room temperature for 30 min. Analysis by HPLC (Superose-12, Pharmacia) showed a shift of about 20% of the radioactivity to the avidin fraction for N_2S_2-3, and that ^{99m}Tc label was on phenyl-MAG_2-biotin. This shows that at least one NHS ester was prepared, that conjugation to biocytin occurred in this case and that the conjugate was labled with ^{99m}Tc.

TABLE 5



General structures of N_3S bifunctional chelators

Tripeptide	R_1	R_2	R_3
Ala-Ala-Ala	CH_3	CH_3	CH_3
Ala-Gly-Gly	CH_3	H	H
Ala-Leu-Ala	CH_3	$(CH_3)_2CHCH_2$	CH_3
Ala-Leu-Gly	CH_3	$(CH_3)_2CHCH_2$	H
Gly-Ala-Ala	H	CH_3	CH_3
Gly-Gly-Ala	H	H	CH_3
Gly-Gly-Gly	H	H	H
Gly-Gly-Ile	H	H	$CH_3CH_2(CH_3)CH$
Gly-Gly-Leu	H	H	$(CH_3)_2CHCH_2$
Gly-Gly-Phe	H	H	$CH_2C_6H_5$
Gly-Gly-Val	H	H	$CH_3(CH_3)CH$
Gly-Leu-Ala	H	$(CH_3)_2CHCH_2$	CH_3
Gly-Leu-Phe	H	$(CH_3)_2CHCH_2$	$CH_2C_6H_5$
Gly-Phe-Ala	H	$CH_2C_6H_5$	CH_3
Gly-Phe-Leu	H	$CH_2C_6H_5$	$(CH_3)_2CHCH_2$
Gly-Phe-Phe	H	$CH_2C_6H_5$	$CH_2C_6H_5$
Gly-Phe-Ser	H	$CH_2C_6H_5$	CH_2OH
Gly-Pro-Ala	H	CH_2CH_2	CH_3
Gly-Ser-Phe	H	CH_2OH	$CH_2C_6H_5$
Ile-Pro-Ile	$CH_3CH_2(CH_3)CH$	CH_2CH_2	$CH_3CH_2(CH_3)CH$
Leu-Gly-Gly	$(CH_3)_2CHCH_2$	H	H
Leu-Gly-Phe	$(CH_3)_2CHCH_2$	H	$CH_2C_6H_5$
Met-Leu-Phe	$CH_3SCH_2CH_2$	$(CH_3)_2CHCH_2$	$CH_2C_6H_5$
Phe-Phe-Gly	$CH_2C_6H_5$	$CH_2C_6H_5$	H
Phe-Phe-Phe	$CH_2C_6H_5$	$CH_2C_6H_5$	$CH_2C_6H_5$
Pro-Gly-Gly	CH_2CH_2	H	H
Ser-Ser-Ser-	CH_2OH	CH_2OH	CH_2OH
Thr-Val-Leu	CH_3CHOH	$CH_3(CH_3)CH$	$(CH_3)_2CHCH_2$
Val-Gly-Gly	$CH_3(CH_3)CH$	H	H
Val-Pro-Leu	$CH_3(CH_3)CH$	CH_2CH_2	$(CH_3)_2CHCH_2$

R_4 is CH_3 and H for each compound respectively.

Preliminary stability studies in serum have been performed with the phenyl-MAG_2-biotin-^{99m}Tc. Very little change in the HPLC profile was seen over 24 hrs at 37 °C in serum, and a high molecular weight peak signifying serum protein binding was seen. It is believed that the increased lipophilicity introduced by the addition of a phenylalanine is responsible for the increased protein binding.

A preliminary cysteine challenge was performed at a 500:1 molar ratio of cysteine: biotin for 1 hr at 37°C. Analysis was by Sep-Pak chromatography in which labeled cysteine elutes in the 0.001 M HCl fraction while the labeled biotin elutes in the ethanol/saline fraction. About 52% of the radioactivity was determined to be present as labeled cysteine under the conditions of this measurement. Thus, the stablility of this chelator toward cysteine transchelation appears similar to that of MAG_3-biotin.

Bifunctional chelators with desired properties (e.g., stability to cysteine challenge, lipophilicity, and ability to achieve high specific activities) can be evaluated as conjugates to antibodies, PNA and peptides radiolabeled with chelated $^{99m}Tc$by the methods described herein, or other methods known in the art. Thus, a variety of chelators can be rapidly synthesized and tested to determine optimal characteristics.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

The contents of all patents and publications referred to herein are hereby incorporated by reference.

Other embodiments are within the following claims.

What is claimed is:

1. A composition comprising:
a nucleic acid;
an oligopeptide tetradentate chelator moiety; and
a radionuclide, wherein the chelator moiety is covalently linked to the nucleic acid, and the radionuclide is complexed with the chelator moiety.

2. The composition of claim 1, wherein the nucleic acid is selected from the group consisting of deoxyribonucleic acid, ribonucleic acid, phosphorothioate nucleic acid, and peptide nucleic acid.

3. The composition of claim 1, wherein a portion of the nucleic acid sequence is complementary to a nucleic acid sequence expressed specifically by tumor cells.

4. The composition of claim 1, wherein the nucleic acid is covalently bound to the chelator moiety through a nitrogen atom of the nucleic acid.

5. The composition of claim 4, wherein the chelator moiety comprises at least one sulfur atom.

6. The composition of claim 1, wherein the radionuclide is selected from the group consisting of technetium-93m, technetium-95m, technetium-99m, rhenium 186, rhenium 188 and rhenium 189.

7. The composition of claim 1, wherein the radionuclide is technetium-99m.

8. A pharmaceutical composition comprising a composition of claim 1, in a pharmaceutically acceptable carrier.

9. A composition comprising an activated ester of an acetyl-protected mercaptoacetyl oligopeptide chelator.

10. The composition of claim 9, wherein the activated ester is an N-hydroxysuccinimide ester.

11. The composition of claim 9, wherein the chelator is biotinylated.

12. The composition of claim 9, wherein at least one residue of the oligopeptide is selected from the group consisting of glycine, serine, threonine, proline, alanine, methionine, valine, isoleucine, tryptophan, tyrosine, and phenylalanine.

13. A composition comprising an [N-[N-[N-mercaptoacetyl]]]-tripeptide, wherein a sulfur atom of said tripeptide is protected by a protecting group of the formula -C(O)-lower alkyl.

14. The composition of claim 13, wherein the tripeptide is triglycine or triserine.

15. A composition comprising:

a polymer moiety; and

a tetradentate mercaptoacetyl oligopeptide chelator moiety comprising a sulfur atom protected by a protecting group of the formula -C(O)-lower alkyl, said chelator being covalently linked to the polymer moiety, said linkage being other than a thiourea.

16. The composition of claim 15, wherein the polymer moiety is a protein.

17. The composition of claim 16, wherein the protein is selected from the group consisting of an antibody, an antibody fragment, and a binding protein.

18. The composition of claim 16, wherein the protein is selected from the group consisting of avidin and streptavidin.

19. The composition of claim 15, wherein said polymer moiety is a nucleic acid sequence.

20. The composition of claim 15, wherein the chelator moiety comprises a sulfur atom protected by an acetyl protecting group.

21. A pharmaceutical composition comprising a composition of claim 15, in a pharmaceutically acceptable carrier.

22. A composition comprising:

a targeting moiety; and

a tetradentate mercaptoacetyl oligopeptide chelator moiety comprising a sulfur atom protected by a protecting group of the formula -C(O)-lower alkyl, said chelator being covalently linked to the targeting moiety.

23. The composition of claim 22, wherein the targeting moiety is a biotin compound.

24. A method of preparing ing an activated ester of an S-protected mercaptoacetyl oligopeptide comprising the steps of:

contacting an oligopeptide with an activated ester of an S-protected thioglycolic acid under conditions such that an S-protected mercaptoacetyl oligopeptide and an activating alcohol are formed; and

contacting reacting the S-protected mercaptoacetyl oligopeptide and the activating alcohol with a coupling reagent under conditions such that an activated ester of an S-protected mercaptoacetyl oligopeptide is formed.

25. The method of claim 24, wherein the coupling reagent is dicyclohexylcarbodiimide.

26. The method of claim 24, wherein step (b) further comprises providing additional activating alcohol.

27. The method of claim 24, wherein the oligopeptide is a tripeptide.

28. A method of forming under mild conditions a polymer-chelator-radionuclide complex, comprising the steps of:

contacting a polymer-chelator composition with a radionuclide, said chelator being a tetradentate mercaptooligopeptide comprising a sulfur atom protected by a protecting group of the formula -C(O)-lower alkyl; and

allowing a polymer-chelator-radionuclide complex to form under mild conditions.

29. The method of claim 28, wherein the polymer is a nucleic acid.

30. The method of claim 28, wherein the radionuclide is technetium-99m.

31. The polymer-chelator-radionuclide complex formed by the method of claim 28.

32. The composition of claim 31 in a pharmaceutically acceptable carrier.

33. A method of preparing a polymer-chelator composition in which the chelator comprises a sulfur atom protected by a protecting group of the formula -C(O)-lower alkyl, the method comprising:

contacting a polymer with an active ester of a chelator, said chelator comprising a sulfur atom protected by a protecting group of the formula -C(O)-lower alkyl, under conditions such that a polymer-chelator-C(O)-lower alkyl composition is prepared.

34. The method of claim 33, in which the polymer is a protein.

35. The method of claim 33, in which the polymer is a nucleic acid.

36. A kit comprising a composition of claim 1, a container, and instructions for use.

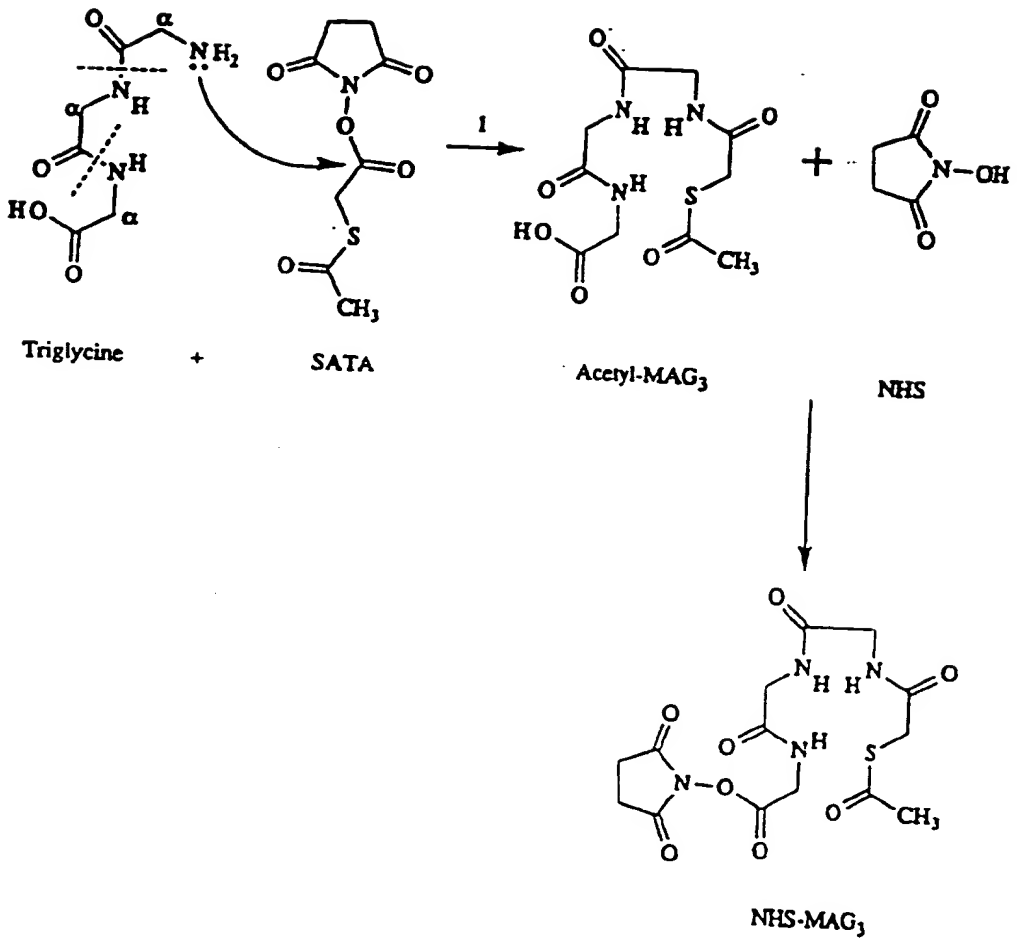


FIGURE 1

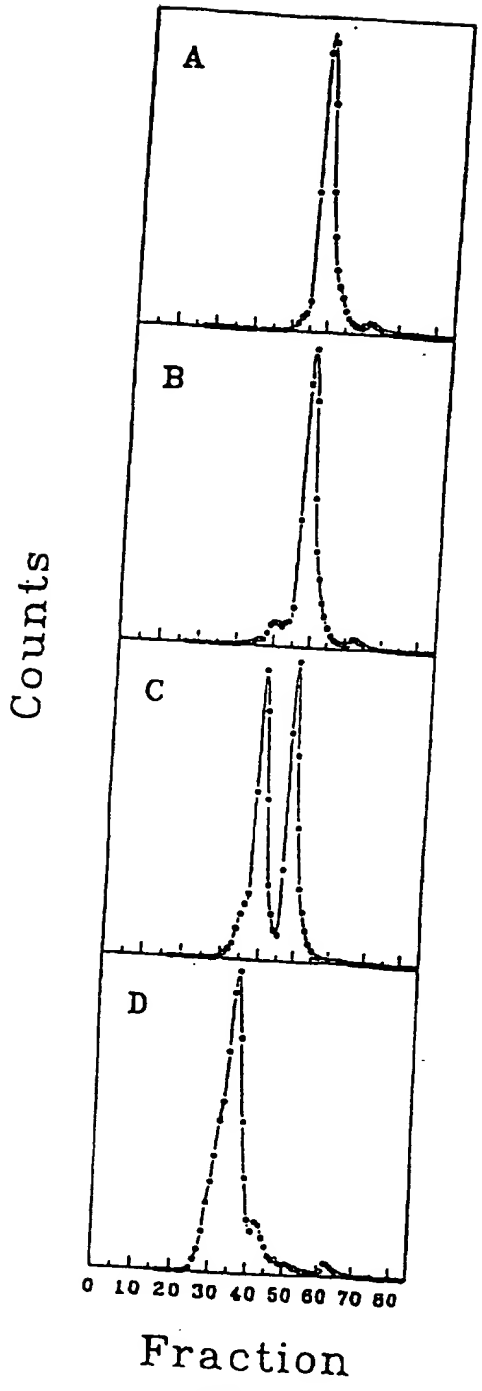


FIGURE 2

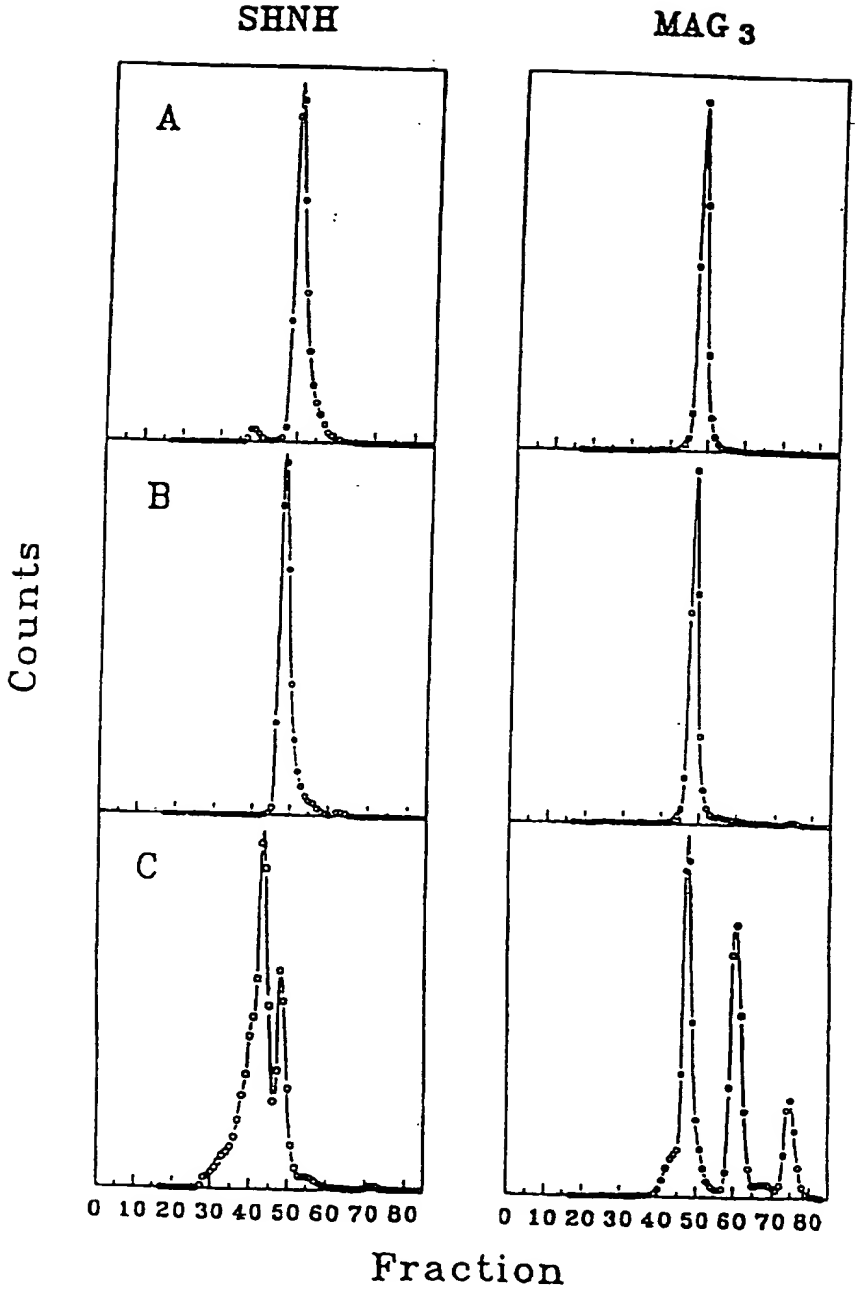

SHNH
MAG 3
A
B
C
Counts
0 10 20 30 40 50 60 70 80
0 10 20 30 40 50 60 70 80
Fraction

FIGURE 3

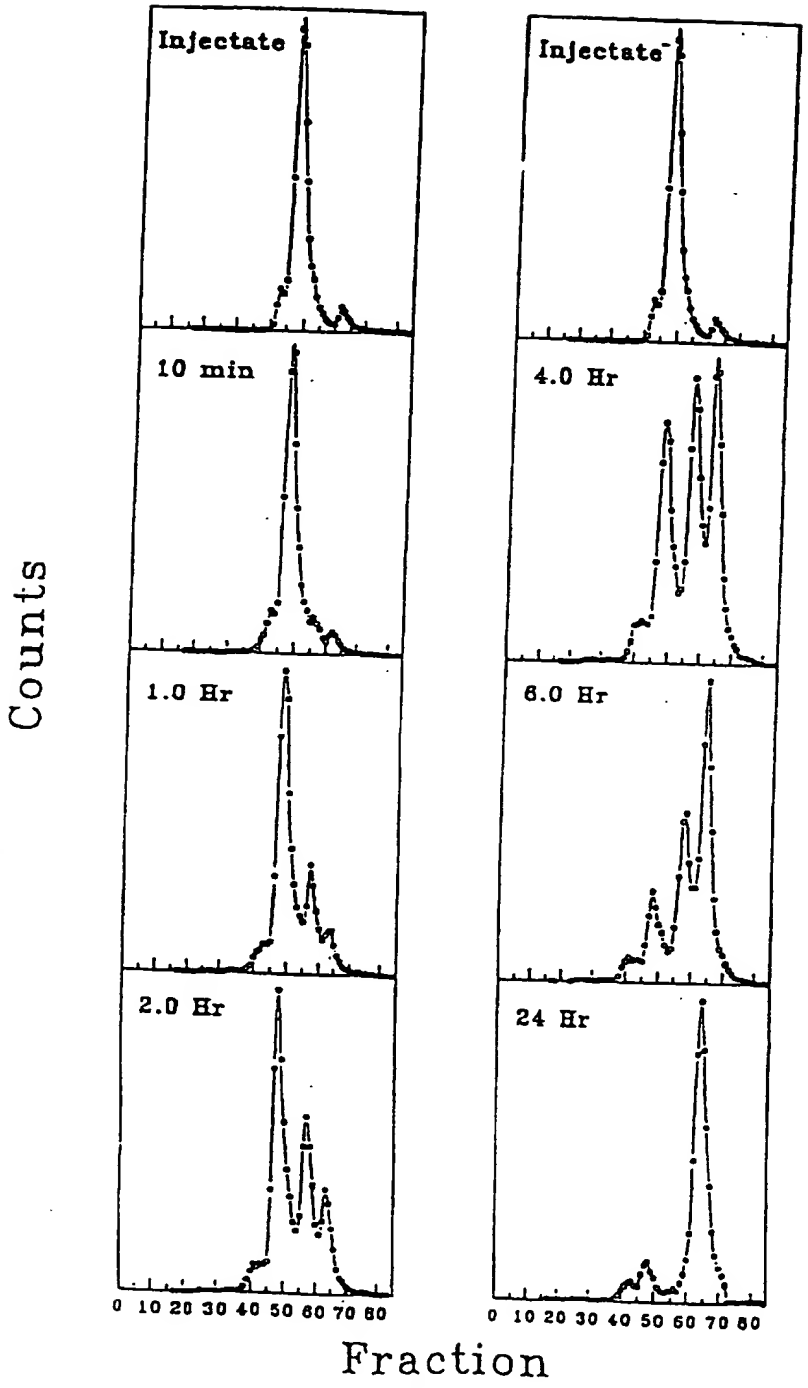

Injectate
Injectate
10 min
4.0 Hr
1.0 Hr
6.0 Hr
2.0 Hr
24 Hr
Counts
0 10 20 30 40 50 60 70 80
0 10 20 30 40 50 60 70 80
Fraction

FIGURE 4

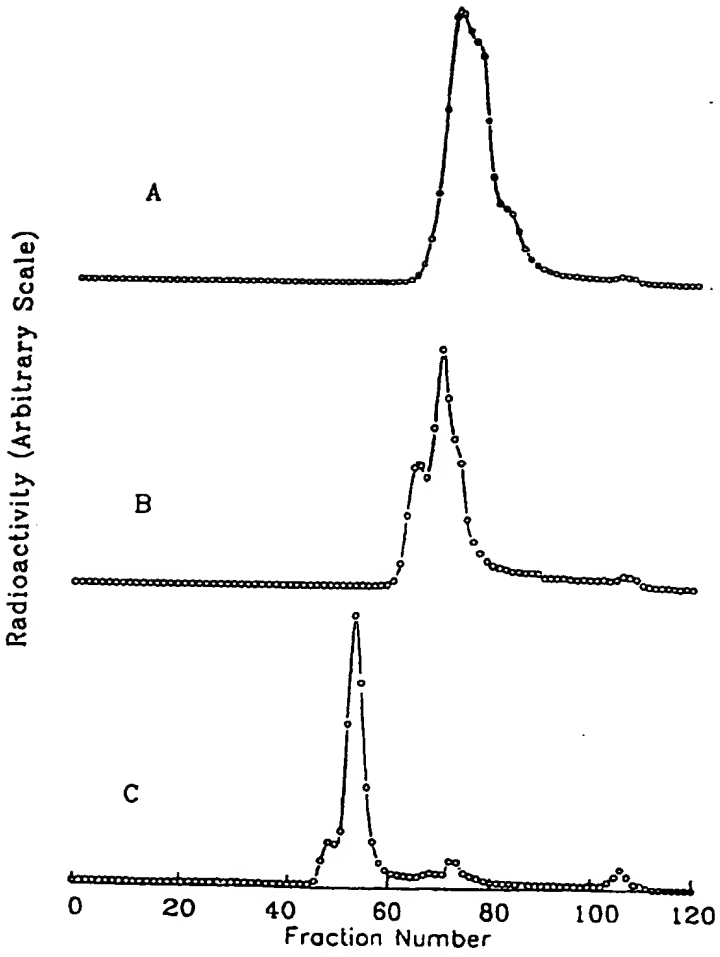

A
B
C
Radioactivily (Arbitrary Scale)
0
20
40
60
80
100
120
Fraction Number

FIGURE 5

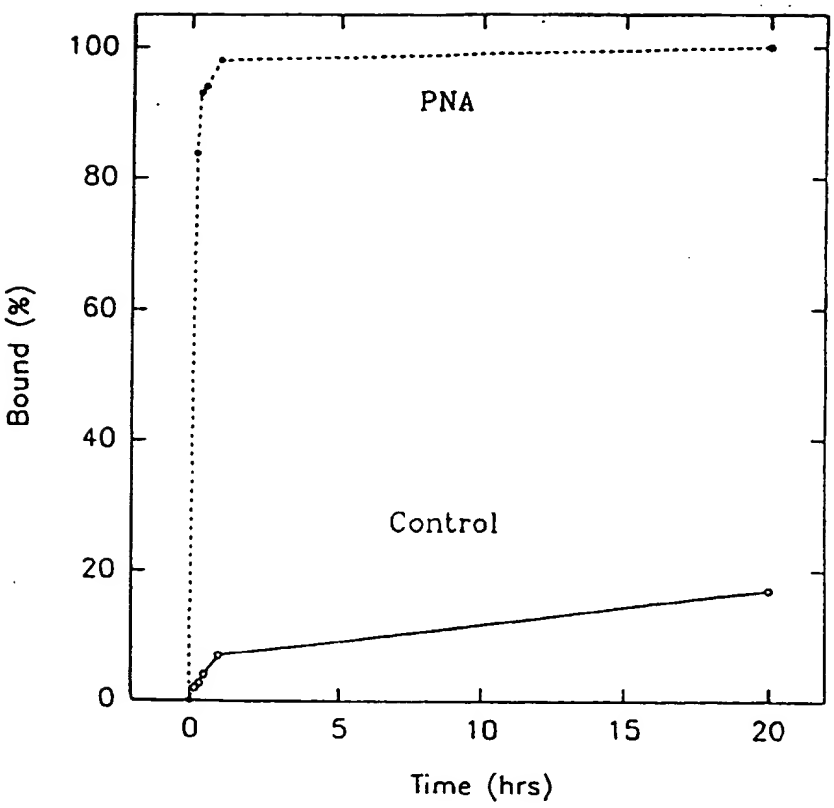


FIGURE 6

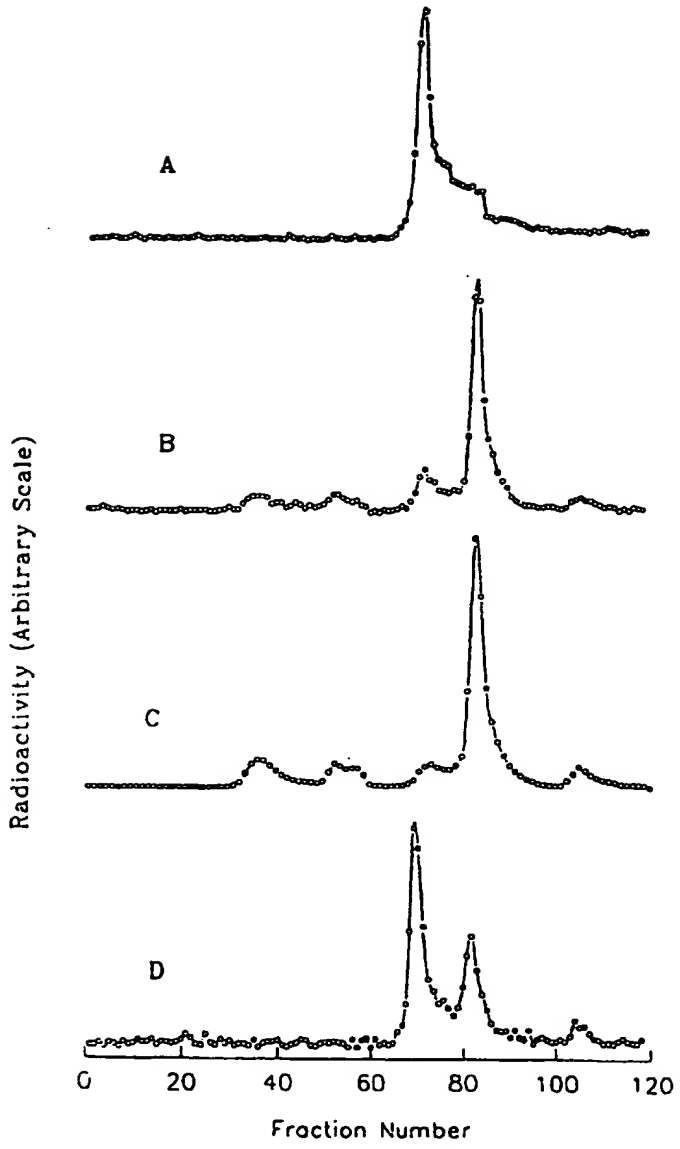

A
B
C
D
Radioactivity (Arbitrary Scale)
0
20
40
60
80
100
120
Fraction Number

FIGURE 7

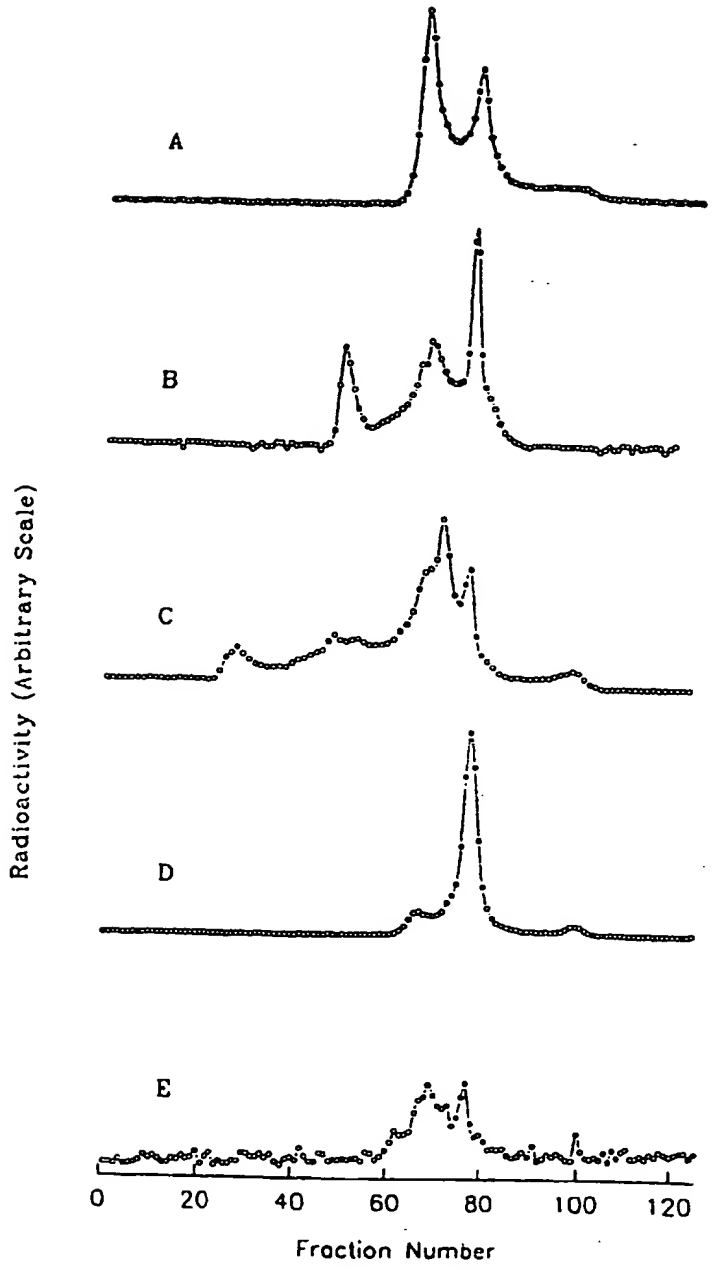

A
B
C
D
E
Radioactivity (Arbitrary Scale)
0
20
40
60
80
100
120
Fraction Number

FIGURE 9

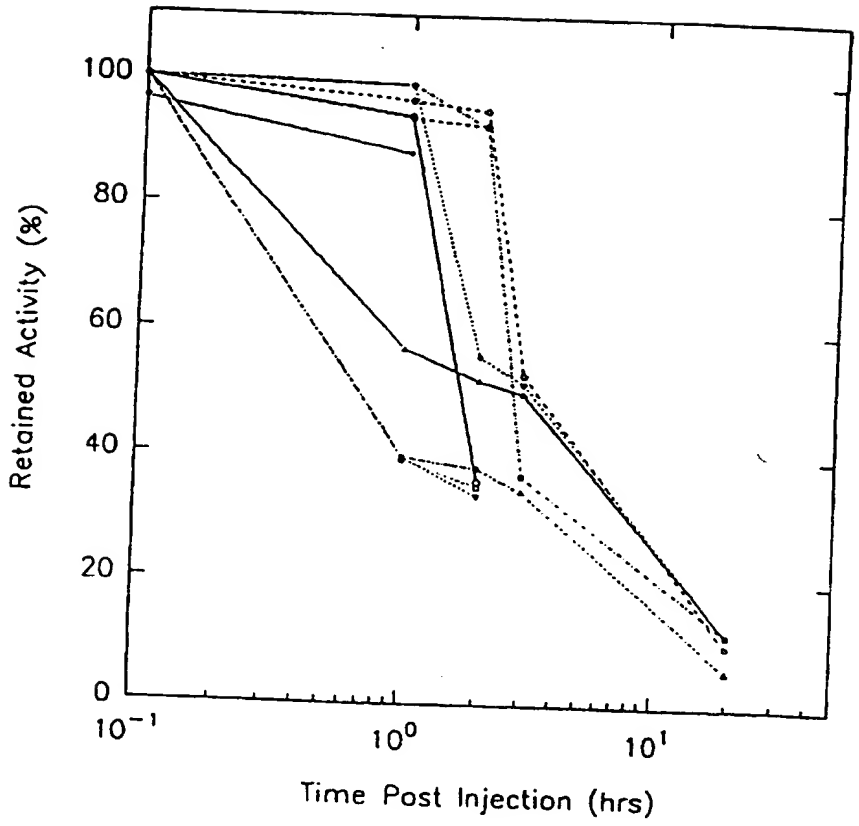

100
80
60
40
20
0
Retained Activity (%)
10^{-1}
10^{0}
10^{1}
Time Post Injection (hrs)

FIGURE 10

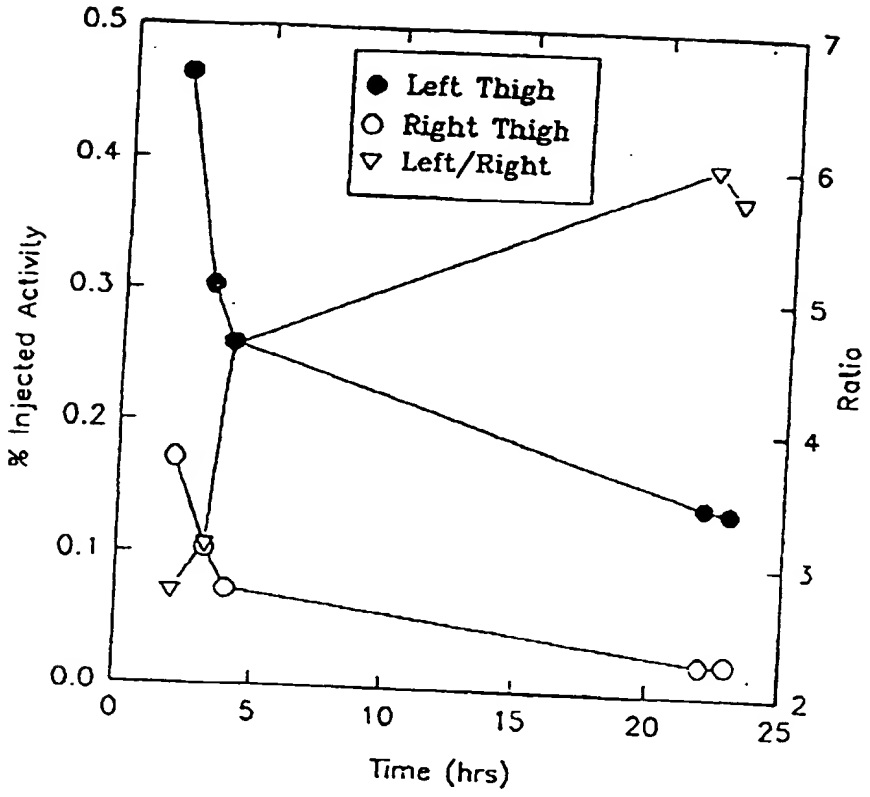

0.5
0.4
0.3
0.2
0.1
0.0
% Injected Activity
0
5
10
15
20
25
Time (hrs)
7
6
5
4
3
2
Ratio
Left Thigh
Right Thigh
Left/Right

FIGURE 11

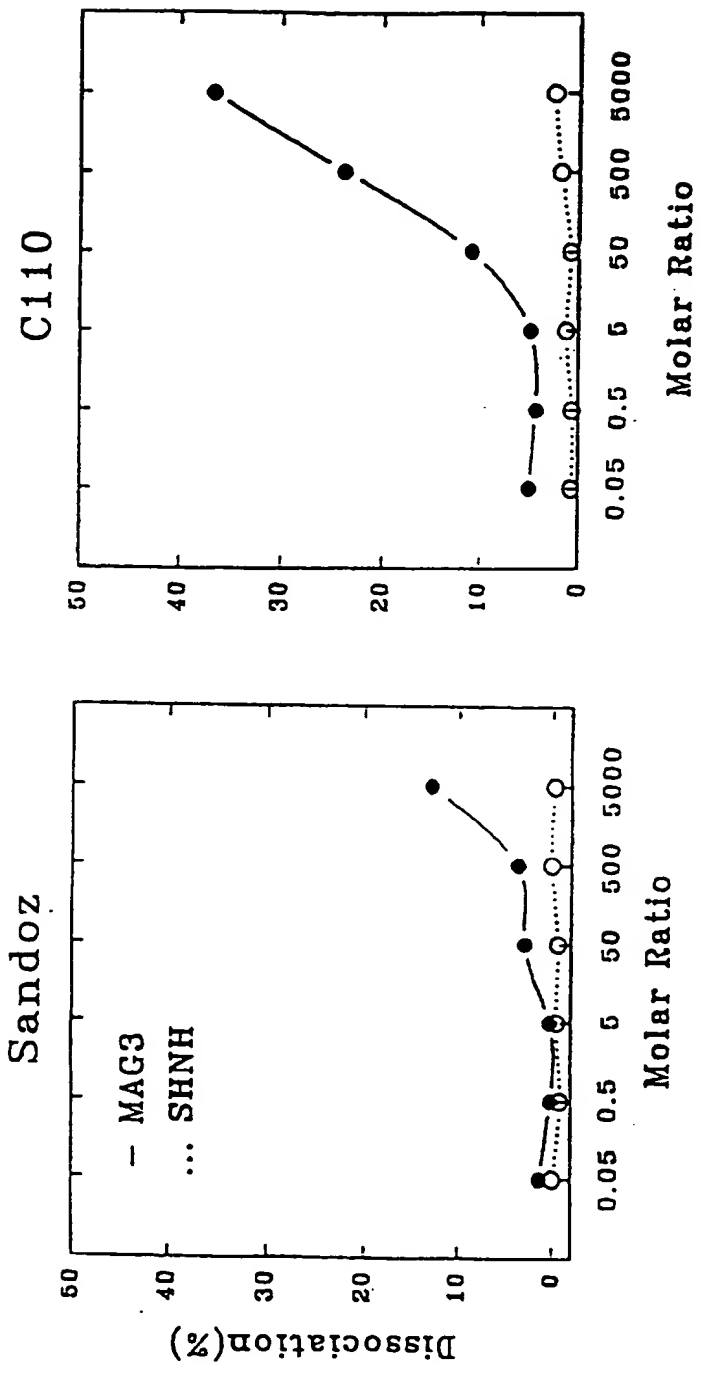

Sandoz
C110
— MAG3
... SHNH
Dissociation(%)
Molar Ratio
Molar Ratio
0.05 0.5 5 50 500 5000
0 10 20 30 40 50

FIGURE 12

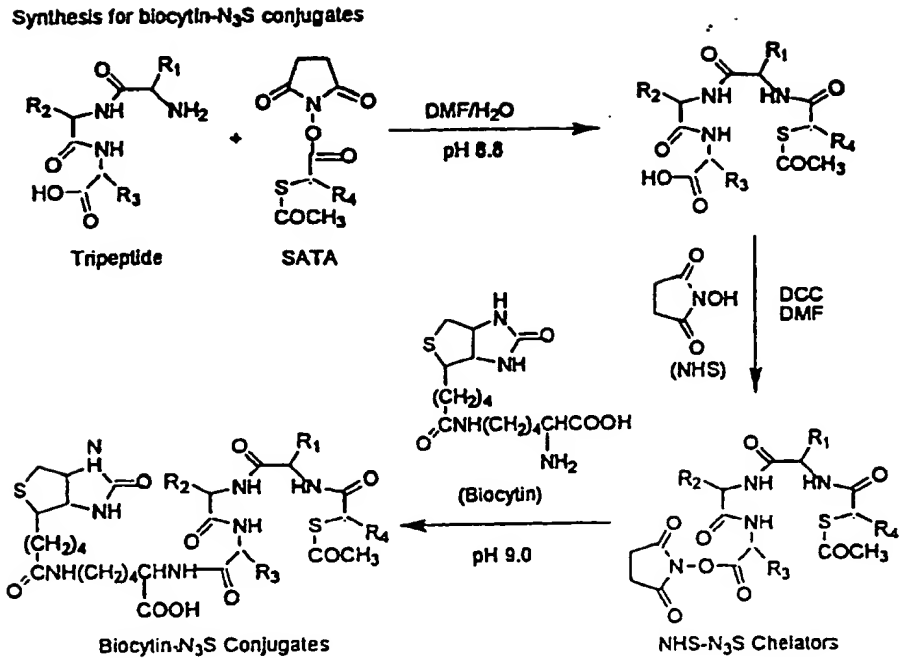
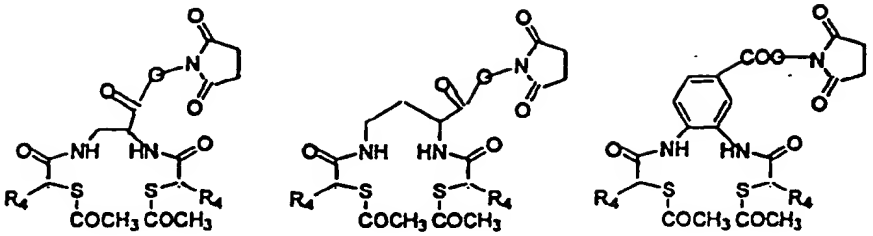

Synthesis for biocytin-N3S conjugates
Tripeptide
SATA
DMF/H2O
pH 8.8
(NHS)
DCC
DMF
(Biocytin)
pH 9.0
Biocytin-N3S Conjugates
NHS-N3S Chelators

FIGURE 13

Structures of N_2S_2 bifunctional chelators



Synthesis for N_2S_2-1 Conjugates

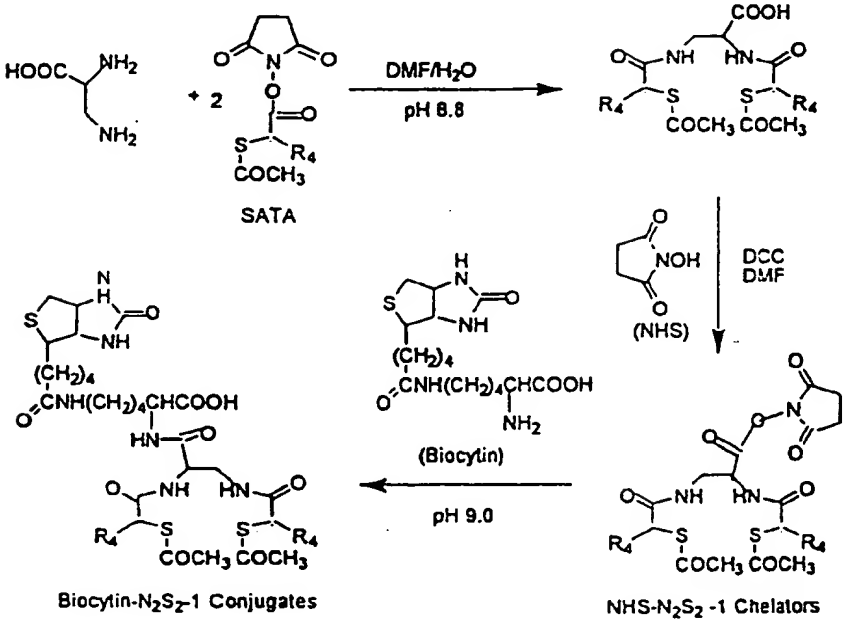


FIGURE 14

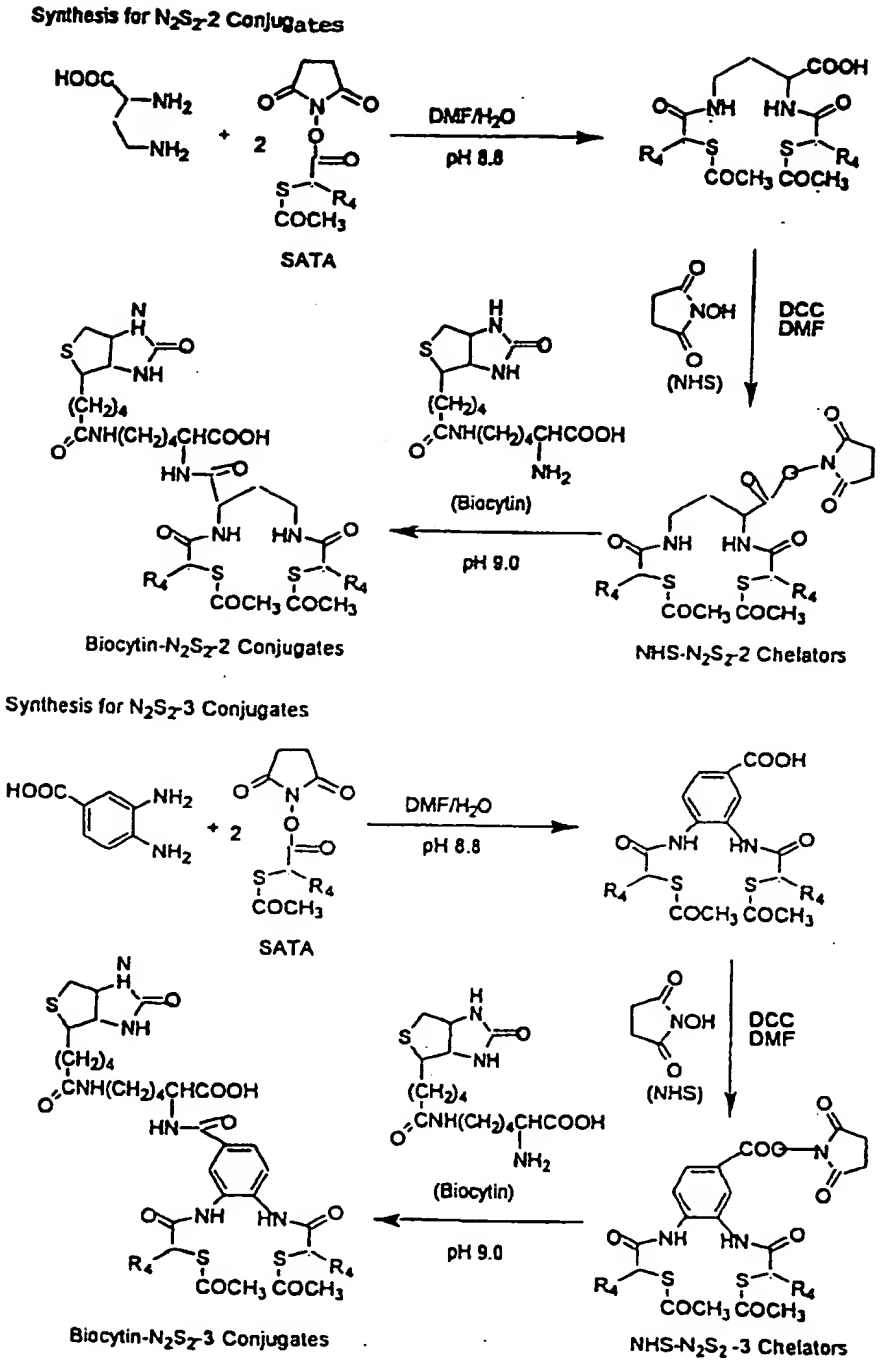

Synthesis for N_2S_2-2 Conjugates
SATA
DMF/H2O
pH 8.8
(NHS)
DCC
DMF
(Biocytin)
pH 9.0
Biocytin-N_2S_2-2 Conjugates
NHS-N_2S_2-2 Chelators
Synthesis for N_2S_2-3 Conjugates
SATA
DMF/H2O
pH 8.8
(NHS)
DCC
DMF
(Biocytin)
pH 9.0
Biocytin-N_2S_2-3 Conjugates
NHS-N_2S_2-3 Chelators

FIGURE 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/04052

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 51/00; A61M 36/14
US CL :424/1.69
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/1.11, 1.65, 1.69, 1.73, 9.1; 534/14; 530/300

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

USPATFUL, BIOSIS, MEDLINE, EMBASE, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WINNARD P. Jr. ET AL A method for the facile preparation of MAG3 conjugated DNA used as an alternative 99m-Tc labeling method to avoid nonspecific protein binding. Eleventh International Symposium on Radiopharmaceutical Chemistry, abstract, 13-17 August 1995.	1-36
Y	HNATOWICH D.J. ET AL Directly and indirectly technetium-99m labeled antibodies-a comparison of in vitro and animal in vivo properties. J. Nucl. Med., January 1993. Vol. 34, No. 1, pages 109-119, especially, abstract and page 110, "Indirect labeling".	1-36

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 MAY 1997	24 June 1997 (24.06.97)
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DAMERON JONES Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/04052

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HNATOWICH D.J. ET AL Comparative Properties of a technetium-99m labeled single-stranded natural DNA and a phosphorothioate derivative in vitro and in mice. J. Pharmacology and Experimental Therapeutics 1996, Vol. 276, No. 1, pages 326-334, especially abstract and page 327, Figure 1 and "Materials and methods".	1-36
Y	EISENHUT M. ET AL Bifunctional NHS-BAT ester for antibody conjugation and stable technetium-99m labeling: conjugation chemistry, immunoreactivity, and kit formulation, J. Nucl. Med., February 1996Vol. 37, No. 2, pages 362-370, especially, abstract and pages 363-364, "Materials and methods".	1-36
Y	DEWANJEE M. ET AL Noninvasive imaging of c-myc oncogene messenger RNA with indium-111-antisense probes in a mammary tumor bearing mouse model, 1994, J. Nucl. Med., Vol. 35, No. 6, pages 1054-1063, especially, abstract page 1055, Figure 1 and pages 1055-1057, "Materials and methods".	1-36
Y	DEWANJEE M. Radiolabeled antisense probes: diagnosis and therapy, Diagn. Oncol., 1993, Vol. 3, pages 189-208, especially, abstract, page 191, Figure 2.	1-36